REMARKS/ARGUMENTS

The office action of November 25, 2009 has been carefully reviewed and these remarks are responsive thereto. Reconsideration and allowance of the instant application are respectfully requested. Claims 1-16 remain in this application. Claim 17 has been canceled without prejudice or disclaimer.

Affirmation of Restriction Requirement

Applicants confirm the election of claims 1-16. Claim 17 has accordingly been canceled. Claim 1 has also been amended in view of the elected species. The only remaining heterocyclic groups or heteroaromatic groups are those in the position of the aryl group at the 2, 5-dihydro-pyrazol moiety in the compound of formula (IA) or (IB). With respect to this aryl group, in the elected species, Ar is a 2-furyl and thus a heteroaromatic group.

Rejections under 35 U.S.C. § 112, first paragraph

Claim 16 stands rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. This rejection is respectfully traversed.

Claim 16 has been amended to reflect that the conditions are selected from rheumatoid arthritis, psoriasis, multiple sclerosis, and diabetes. Support for these conditions is found in paragraphs 75, 71, 62, and 44 of the corresponding US published application (20070213345).

The aforementioned diseases are well-known autoimmune diseases for which treatment by the suppression of T cell activation is relevant. In T cell activation the central role is attributed to CD80/CD28 interaction. According to the data contained in the present invention the claimed compounds are qualified binders to CD80 (see table 1, in particular compounds 5, 9-15, 17-18, 26-27, 30, and 32-33, and paragraphs [0167], [0177] and [0178] of the homogenous time resolved fluorescence assay (HTRF assay). In this context, reference is made to the 2nd sentence of paragraph [0173]: "*the compounds of examples 7, 11 & 18-21 have greater affinity and longer residence times on CD80 than CD28, having K_DS of less than 100 nM, and off-rates of $2*10^{-2}$ indicating that the pyrazolones will be able to compete effectively with the endogenous ligand.*"

Therefore, the present application itself already contains convincing data that the claimed compounds are suitable for treating the autoimmune disease mentioned in amended claim 16. Furthermore, at the priority date of this application, which is November 4, 2003, it was known to the person skilled in the art that activated T cells inappropriately attack and destroy host cells of various tissues in autoimmune diseases.[1]

It was also known that T cell activation requires a co-stimulatory signal generated by the interaction of CD80 (a molecule on the surface of antigen-presenting cells) and CD28 (a molecule on the surface of T cells).[2] Therefore, at the time this application was filed, it was reasonable for one skilled in the art to consider that an agent which blocks the CD80/CD28 interaction would prevent or reduce activation of T cells, thereby reducing the damage caused by activated T cells in autoimmune diseases. In fact, many research groups have explored and continue to explore agents which act to suppress T cell activation. The relevance of suppressing T cell activation for treating autoimmune diseases is confirmed by post-filing date references, such as those provided in the IDS for rheumatoid arthritis,[3] psoriasis,[4] multiple sclerosis[5] and type 1 diabetes.[6]

Representative examples of such agents are HMG CoA reductase inhibitors[7] and tyrosine kinase inhibitors.[8] Other examples of such agents already are on the market, such as for example anti-TNF (tumor necrosis factor) antibodies which inhibit one of the major T cell pro-inflammatory cyokines. Anti-TNF antibodies include infliximab (REMICADE®), a dalimumab (D2E7/HUMIRA®) and) etanercept (ENBREL®).[9] More recently, abatacept (ORENCIA®), a protein which inhibits co-receptor signaling on T cells, has also reached the market showing good efficacy in rheumatoid arthritis.[10]

The following articles are attached to this response.

[1] see Kobata et al., *Rev. Immunogenet.* 2000, 2(1), 74-80.
[2] see Lenschow et al., *Ann. Rev. Immunol.* 1996, 14, 233-258, cited in par. 3 of the specification; see also Linsley et al. *J. Exp. Med.*, 1991, 173, 721-730; Dubey et al., *J. Immunol.*, 1995, 155, 45-47; and Suresh et al., *J. Immunol.*, 2001, 167, 5565-5573.
[3] see Cope et al., *Clin. Exp. Rheumatol.* 2007, 24, 4-11.
[4] see Choy, Curr. *Rheumatol. Rep.* 2007, 6, 437-441.
[5] see Weiss et al., *Neuroimmunol.* 2007, 191, 79-85.
[6] see Mallone and Endert, *Curr. Diab. Rep.* 2008, 8, 101-106.
[7] see Brumenau et al., *Clin. Immunol.* 2006, 119, 1-12.
[8] see Appel and Brossart, *Endocri. Metab. Immune Disord. Drug Targets,* 2007, 7, 93-97.
[9] see Kristensen et al., Scan. *J. Rheumatol.* 2007, 36, 411-417.
[10] see Chitale and Moots, *Expert. Opin. Biol. Ther.* 2008, 8, 115-122.

Claim 16 is in full compliance with the enablement requirement. Withdrawal of the present rejection is requested.

CONCLUSION

It is believed that no fee is required for this submission. If any fees are required or if an overpayment is made, the Commissioner is authorized to debit or credit our Deposit Account No. 19-0733, accordingly.

All rejections having been addressed, applicants respectfully submit that the instant application is in condition for allowance, and respectfully solicit prompt notification of the same.

Respectfully submitted,

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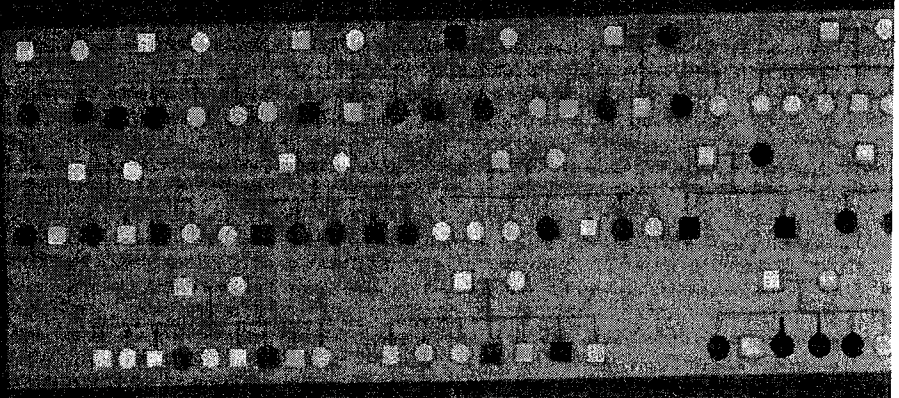

Genetics and Immunobiology of Autoimmune disease

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Role of costimulatory molecules in autoimmunity

Key words:
APC-T cell interaction; costimulatory molecule; self-reactive T cell; tolerance

Abstract: The precise mechanisms by which self-reactive T cells are activated and tolerance to self-antigens is broken are still not fully understood. It is widely accepted that dysregulation of costimulation contributes to the initiation and maintenance of autoimmunity due to activation of self-reactive T cells. Many of the costimulatory molecules thought to be essential for T cell activation have been identified. The CD28/CD152 (CTLA-4)-CD80/CD86 and CD40-CD154 (CD40 ligand) interactions are such receptor/ligand pairs that have been shown to be important in interactions between antigen-presenting cells and T cells. *In vivo* studies using costimulatory molecule-specific antibodies and fusion proteins and genetically manipulated animals have greatly increased our understanding of the role of these costimulatory molecules in the regulation of cellular processes that lead to autoimmunity and resultant autoimmune diseases.

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Optimal T cell activation requires two signals (1). Signal 1 is the occupation of the T cell receptor (TCR) by a complex formed between an antigen peptide and the major histocompatibility complex (MHC) molecules on the surface of the antigen-presenting cell (APC). The second (costimulatory) signal is delivered by molecules on the APC surface. Signal 1 is therefore antigen-specific whereas signal 2 is non-antigen specific. The most important costimulatory signals for T cell activation are cell-cell interactions through membrane-bound ligand/receptor pairs.

One of the most important events in the development of autoimmune disease is the activation of self-reactive T cells (2). The precise mechanisms by which self-reactive T cells are activated and tolerance to self-antigens is broken are not yet fully understood. There is abundant evidence for dysregulation of costimulation as one of the mechanisms involved in autoimmunity. Over the past several years, the use of genetically manipulated animals and *in vivo* administration of antibodies and fusion proteins specific for costimulatory molecules have provided us with an understanding of the molecular

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mechanisms by which an autoimmune response is initiated and continues through the dysregulation of costimulation (Table 1).

Positive regulation of self-reactive T cell activation by costimulatory molecules

Since costimulatory signals are important factors in determining whether presentation of antigens to T cells leads to activation or anergy, it has been suggested that costimulatory molecules are involved in the regulation and activation of self-reactive T cells. Several ligand/receptor pairs have been shown to provide costimulatory functions involving activation of T cell proliferation and production of effector cytokines after TCR signaling (3). Two of these ligand/receptor pairs, CD80/CD86-CD28/CD152 (CTLA-4) and CD154 (CD40 ligand)-CD40 have received considerable attention. These interactions have been shown to be essential for costimulation at the immunological synapse between APCs and T cells because CD80/CD86-CD28 interactions play a crucial role in antigen presentation and prevent induction of anergy (4, 5), while CD154-CD40 interactions are required for the activation of APCs (6). When CD4 T cells are primed by antigen presented on APCs, they upregulate CD154 on the cell surface. In turn, they upregulate CD80/CD86 on APCs via CD40 (5). Finally, CD80/CD86 activates T cells via CD28. Thus, CD154-CD40 interactions control the expression of costimulatory molecules such as CD80/CD86 on APCs, and regulation at this step may provide a safeguard to help prevent activation of self-reactive T cells and autoimmune responses (7).

Windhagen et al. (8) showed that early multiple sclerosis (MS) lesions in the human central nervous system were accompanied by upregulation of costimulatory molecules such as CD80 and CD86. *In vitro* experiments have shown that myelin-specific T cells from healthy individuals require CD80/CD86 costimulation, whereas T cells from MS patients are already activated *in vivo* and are costimulation-independent (9). Overexpression of CD80 has also been detected on B cells and monocytes in synovial fluid in patients with rheumatoid arthritis (RA) (10) and in those with autoimmune thyroiditis (11). CD154 is upregulated on T and B cells in patients with systemic lupus erythematosus (SLE) and in a murine model of SLE, BXSB (12–14). Taken together, these studies suggest that costimulatory molecules such as CD80 and CD154 are upregulated in a number of autoimmune diseases, and that this process may be required for the activation of self-reactive T cells.

Based on these observations, CD152-Ig, which blocks the interaction between CD28 and its ligands CD80/CD86, has been used in the treatment of experimental autoimmune encephalomyelitis (EAE), a model for MS, to successfully inhibit T cell activation and induce immunological tolerance *in vivo* (15, 16). Addition of CD152-Ig also blocked autoantibody production and prolonged life in the New Zealand Black/White (NZB/NZW) F1 lupus mouse model (17). However, CD152-Ig or anti-CD86 antibody (Ab) inhibited the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse, a model for insulin-dependent diabetes mellitus (IDDM), while anti-CD80 Ab accelerated it (18). Furthermore, anti-CD80 Ab, but not anti-CD86 Ab, inhibited the development of EAE (19) and blocked epitope spreading during remission, resulting in long-term inhibition of relapses (20). Thus, CD80 and CD86 may have distinct regulatory functions during the development of individual autoimmune diseases, although CD80/CD86-CD28 interactions are closely involved in autoimmune responses.

CD28-deficient mice were highly resistant to collagen-induced arthritis (CIA), a model for RA (21). Oliveira-dos-Santos et al. (22) recently reported that EAE could be induced at high antigen doses in CD28-deficient mice, suggesting that CD28 does not regulate immunological anergy, but rather adjusts the threshold for the activation of self-reactive T cells.

Administration of anti-CD154 Ab significantly prolonged survival, reduced the severity of nephritis and diminished associated inflammation, vasculitis and fibrosis in lupus-prone mice, (SWR/NZB) F1 (23). During the induction of experimental autoimmune myasthenia gravis (EAMG), CD154-deficient mice were completely resistant while CD28-deficient mice became less susceptible (24). The role

Antibodies and fusion proteins used *in vivo*, and genetically altered animals used to understand the role of costimulatory molecules in autoimmunity

	Ref.
Antibody/Fusion protein	
Anti-CD80/CD86 Ab	18–20, 26, 56, 58
Anti-CD152 Ab	46, 59
CD152-Ig	15–18, 27, 30, 36, 59
Anti-CD154 Ab	23, 27, 63
CD134-Ig	30
Anti-ICAM-1/LFA-1 Ab	28
Transgene/null mutation animals	
CD80-transgenic mice	31–34, 65
CD28-deficient mice	21, 22, 24, 25, 50
CD80-deficient mice	35, 36, 44
CD152-Ig-transgenic mice	45, 50
CD154-deficient mice	6, 24, 25
ICAM-1-deficient mice	51

Table 1

of CD154-CD40 interactions in the development of autoimmune diseases was also studied in a defined system where CD154-deficient mice expressing a myelin basic protein (MBP)-specific TCR transgene were used for the EAE model. Although T cells in these mice were more than 95% self-reactive T cells, and these cells were functional *in vitro* in the presence of CD80* APCs, they could not develop MBP-induced EAE (25), confirming that CD154-CD40 interactions have profound effects on the activation of self-reactive T cells by inducing costimulatory activity on APCs *in vivo*. These studies also provided further evidence that this step is involved in the induction of costimulatory molecules such as CD80/CD86 on APCs.

Although blockade of CD80/CD86-CD28 interactions prevents autoantibody production and nephritis, and prolongs survival of the NZB/NZW F1 lupus mouse model (26), the effect is short-lived and chronic treatment is required for sustained immunosuppression. However, brief simultaneous blockade of CD80/CD86-CD28 and CD154-CD40 produces benefits that last long after treatment has been discontinued in NZB/NZW F1 mice (27). These results again support the notion that CD154-CD40 interactions regulate the subsequent process of costimulation mediated by CD80/CD86-CD28 interactions.

On the other hand, complete protection against autoimmune diabetes in NOD mice was induced by blockade of interactions between leukocyte function-associated antigen (LFA)-1 (CD11a/CD18) and intercellular adhesion molecule (ICAM)-1 (CD54) (28). CD134 (OX-40), a member of the TNF receptor family like CD40, is expressed primarily on activated CD4 T cells and mediates a costimulatory signal for them. CD134 is selectively upregulated on encephalitogenic T cells in EAE (29). Recently, Weinberg et al. (30) have demonstrated that blockade of CD134 with CD134 ligand, which is expressed on APCs, reduced the development of EAE.

The availability of transgenic mice has shown, however, that aberrant expression of costimulatory molecules would not solely account for activation of self-reactive T cells and resultant autoimmunity. Transgenically-induced expression of CD80 on cells such as β cells in the pancreas did not induce autoimmune diabetes, although it was sufficient to confer APC function, suggesting that additional factor(s) are required to develop autoimmunity (31–33). Most self-reactive T cells have probably been "tolerized" in the thymus, leaving mainly low-affinity T cells or T cells specific for antigens that are exclusively expressed on peripheral tissue. Moreover, cells in most tissues express low levels of MHC class I, and usually no MHC class II, limiting the level of expression of any given MHC/peptide complex and thereby ensuring that only high-avidity T cells can be activated. Indeed, transgenic mice that also expressed high levels of MHC class II/peptide complex developed diabetes (32, 34). Reciprocally, transgenic mice developed autoimmune diabetes in the presence of a high proportion of specific T cells provided by transgenically-encoded TCR specific for a β cell antigen (33). These results reinforce the notion that a strong MHC class II/antigen peptide/TCR interaction (signal 1) is essential for the initiation of autoimmune responses even if costimulatory molecules are aberrantly expressed on target cells.

Negative regulation of self-reactive T cell activation by costimulatory molecules

The above studies in genetically engineered mice and mice treated with antibodies and fusion proteins specific for costimulatory molecules provided the first conclusive evidence that dysregulation of costimulation can lead to the activation of self-reactive T cells. Contrary to the previous notion that signal 1 through the TCR in the absence of a costimulatory signal would induce peripheral tolerance (34), studies with CD152-deficient mice clearly showed the requirement for costimulation in the maintenance of tolerance (35, 36). Distinct signaling patterns triggered by the binding of CD28 and CD152 costimulatory receptors to their ligands CD80 and CD86 imply additional regulation of the fate of T cells determined by TCR signals (37). After interaction with CD80/CD86, CD28 and CD152 mediate positive and negative signals, respectively, for T cell activation (35, 38, 39), and CD152 is critical for the induction of peripheral T cell tolerance and deletion of self-reactive T cells (40). CD152 is expressed only transiently on the cell surface, primarily after T cell activation (41), implying that T cell activation is regulated by the net balance between *on* and *off* switches (42). Down-regulation of T cell activation *in vivo* may result from the specific recognition of CD80/CD86 by CD152, and not because of a lack of costimulation (43).

In CD152-deficient mice that exhibit defects in cell death mechanisms, lack of induction of T cell death can lead to the development of autoimmunity (44). Indeed, CD152-deficient mice were protected from lymphoproliferative disorders when injected from birth with soluble CD152 which blocks CD28-CD80/CD86 interactions. This suggests that the role of CD152 is mainly to down-regulate at least the clonal expansion of activated T cells (35). This notion was confirmed by studying transgenic mice expressing a CD152-Ig transgene; soluble CD152-Ig prevented rather than facilitated superantigen enterotoxin B-induced T cell anergy (45). In addition, treatment of EAE-resistant mice with anti-CD152 Ab could induce or exacerbate the disease (46).

Lymphocytes of NOD mice are resistant to apoptosis. A major locus that controls the apoptosis-resistance phenotype is linked to the *Idd5* diabetogenic locus on chromosome 1. Interestingly, the

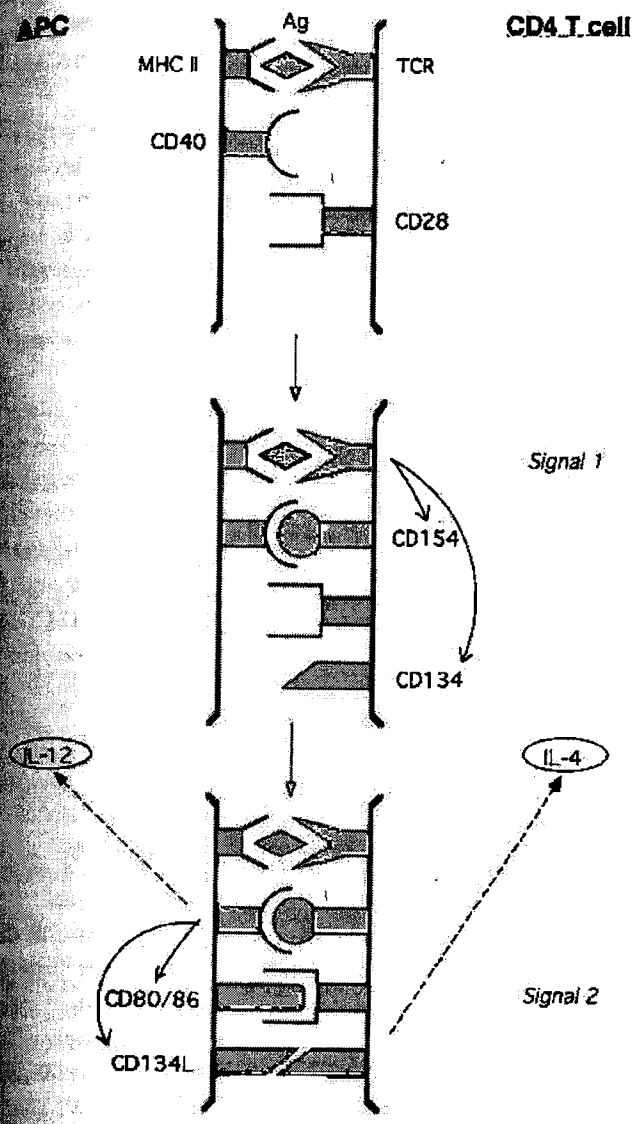

Fig. 1. Initiation of immune response by costimulatory molecules at the immunological synapse. Activation of TCR induces expression of CD154 and CD134 on the CD4 T cell surface. CD154 engagement of CD40 on APCs results in IL-12 production and upregulation of CD80/86 and CD134L expression on the APC surface, which then costimulates CD4 T cells via CD28 and CD134, respectively, subsequently resulting in IL-4 production. At present, it is not clear whether the same APCs and CD4 T cells express both CD134L/IL-12 and CD154/CD134, respectively.

region also contains the *cd152* and *cd28* genes (47). Expression of CD152 on NOD T cells is defective. Accordingly, if CD152-mediated signaling, which normally confers protection from autoimmune disease by maintaining peripheral tolerance, is deficient in NOD T cells, reduced surface expression of CD152 and altered CD152 signaling would induce survival of self-reactive T cells, the breakdown of tolerance and susceptibility to autoimmune disease.

The notion that regulatory T cell anergy induced by impaired costimulation may lead to the pathogenesis of an autoimmune disease is also intriguing. Impaired CD80/CD86 expression on APCs and defects in CD80/CD86-CD28 costimulation underlie abnormal T cell activation in SLE patients (48), and down-regulation of CD28, CD80 and CD86 expression has also been described in T cells and APCs, respectively, from patients with primary biliary cirrhosis (49). CD28-deficient and CD152-Ig transgenic mice promoted the development and progression of spontaneous autoimmune diabetes in NOD mice (50), in marked contrast to the resistance of CD28-deficient mice to induction of experimental autoimmune diseases such as CIA and EAMG (21, 24). The development of EAE was significantly enhanced in ICAM-1-deficient mice, explained by the defect in ICAM-1-mediated down-regulation of autoimmune responses (51).

Role of costimulatory molecules in immune deviation

It is generally thought that organ-specific autoimmune disease results from a preponderance of Th1 cells over Th2 cells. Cytokines such as IFN-γ secreted by Th1 cells activate macrophages and other effector cells, mediating inflammation and tissue injury in target organs. On the other hand, Th2 cells produce cytokines such as IL-4 and IL-10, and suppress the cell-mediated immune response induced by Th1 cells. Cytokines produced by each subset promote growth of their own subset in an autocrine fashion and inhibit the development and function of the other subset. IL-12 and IL-4 play decisive roles in the polarization of T cells, guiding T cell responses towards the Th1 or Th2 phenotype, respectively (52, 53). The principal source of IL-12 is activated APCs such as macrophages and dendritic cells (DCs), whereas IL-4 is initially produced by antigen-stimulated CD4 T cells (Fig. 1). Several experimental models of autoimmune disease have shown that Th2 cytokines may be implicated in the regulation of the activity of self-reactive T cells (54, 55).

Expression of CD80, CD86 and IL-12 has been observed in early MS lesions (8), suggesting that upregulation of CD80 and CD86 induces Th1-type immune responses. In EAE induced with the myelin proteolipid protein (PLP), the pathological process depends on Th1 cells that initiate inflammation within the brain. It is believed that immunization with PLP recruits pathogenic Th1 T cells. When animals were immunized with the same peptide in the presence of anti-CD80 Ab, they were protected from EAE due to the generation of PLP-reactive Th2 cells (19, 56). However, in EAE induced with MBP, MBP-specific Th2 cells had the potential to induce EAE and disease induced by previously activated Th1 cells could not be prevented

by normal lymphocytes or by previously activated Th2 cells (57). In addition, myelin oligodendrocyte glycoprotein- or MBP-induced EAE was significantly enhanced in ICAM-1-deficient mice with reduced Th1 cytokine production and elevated Th2 cytokine production (51).

Orally administered proteins induce systemic hyporesponsiveness to the fed protein. The mechanism underlying oral tolerance depends on the amount of antigen fed, with higher doses inducing deletion and anergy, and lower doses inducing regulatory cells. Orally administered autoantigens suppress many experimental autoimmune diseases. In low-dose oral tolerance to MBP in the EAE model, anti-CD86 Ab augmented TGF-β production and blocked induction of the tolerance (58). In high-dose oral tolerance, CD152-mediated costimulation was required for the induction of peripheral T cell tolerance in antigen-specific TCR transgenic mice (59). It has been recently demonstrated that IL-4 production by naive T cells was highly dependent on CD80/CD86 (60) and that CD152 engagement induced TGF-β production (61), which, in part, contributes to the downregulation of T cell activation.

CD154 expression on T cells is elevated in patients with SLE and RA (12, 13). Because of CD154 upregulation, CD4 T cells can directly induce B cell proliferation leading to autoantibody production, as well as upregulate production of cytokines such as IL-12 from APCs in a contact-dependent manner (62). CD154-deficient mice fail to develop IFN-γ-producing T cells or develop EAE even when carrying a transgene for MBP-specific TCR (25). This phenotype could be normalized by the transfer of $CD80^+$ APCs (25). CD154-deficient mice are also resistant to the development of EAMG (24). Animals treated with anti-CD154 Ab do not develop lupus or CIA (23, 27, 63). Whether these diminished T cell-mediated responses induced by blockade of CD154-CD40 interactions result from diminished cell extravasation, reduced induction of inflammatory cytokines, decreased activation of APC functions, decreased T cell activation, and/or decreased maturation of Th1 cells has not yet been determined.

CD134L is induced on B cells and DCs after CD40 ligation. Using *in vitro* preparations, Flynn et al. (64) recently showed that costimulation of naive CD4 T cells through CD134 promotes IL-4 expression and inhibits IL-12-induced IFN-γ expression, suggesting that CD134 promotes the differentiation of Th2 cells. Thus, the therapeutic effect of CD134-Ig on EAE (30) does not seem to be due to modulation of the balance between Th1 and Th2 cells.

ICAM-1 appears to play a decisive role in the inhibition of IL-4 production, at least in part by APC-T cell interactions. Recent studies by Luksch et al. (65) have demonstrated that coexpression of ICAM-1 and CD80/CD86 on APCs induced little IL-4 from naive T cells, while expression of CD80/CD86 without ICAM-1 on APCs resulted in excessive production of IL-4. This may be in concordance with the notion of aggravated EAE with Th2-polarization in ICAM-1-deficient mice (51).

The strength of MHC class II/antigen peptide/TCR interactions, which depends on the overall avidity of the interactions between APCs and T cells, also controls the profile of cytokine secretion by T cells. The use of different antigen doses *in vitro* and *in vivo* and altered peptide ligands, revealed that lower-avidity interactions result in reduced CD40-CD154 interactions between APCs and T cells, and hence reduced IL-12 production, which appears to favor Th2 cell development (66).

Thus, while under certain circumstances Th2 cells can inhibit autoimmunity by suppressing the functions of self-reactive T cells, under other conditions the same cells can promote autoimmune disease such as EAE and autoimmune diabetes, depending on the system studied. The oversimplification intrinsic to the Th1 pathogenic/Th2 protective paradigm does not appear to fully describe the complexity of the immune response, although the concept of a Th1/Th2 dichotomy is largely valid. Similarly, although it has been considered that each costimulatory molecule affects differentiation of Th1 or Th2 cells, their relationship is still obscure.

Conclusion

The studies discussed above have delineated the central role of APCs in integrating both environmental and accessory signals, which then determine CD4 T cell fate through costimulatory molecules. Antigen presentation and subsequent T cell activation are coordinately regulated at this immunological synapse where CD154-CD40 interactions appear to play a pivotal regulatory role. Thus, the involvement of costimulation and its modulation or dysregulation are directly relevant to activation of self-reactive T cells. In complete agreement on the relationship between costimulatory molecules, Th1/Th2 differentiation and pathogenesis/pathophysiology may reflect the complexity of autoimmunity in each individual disease. One might ask why so many costimulatory molecules have been proposed to account for the immune response or autoimmunity. Perhaps the simplest answer is that the immunological synapse involves so many distinct ligand/receptor interactions, each capable of inducing qualitatively different signaling events, that one requires an extremely complex system for amplification, termination and fine control. After further accumulation of evidence of *in vivo* functions mediated by each costimulatory molecule, manipulating the costimulatory signal will indeed be the goal of therapeutic strategies for autoimmune diseases.

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CD28/B7 SYSTEM OF T CELL COSTIMULATION

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ABSTRACT

T cells play a central role in the initiation and regulation of the immune response to antigen. Both the engagement of the TCR with MHC/Ag and a second signal are needed for the complete activation of the T cell. The CD28/B7 receptor/ligand system is one of the dominant costimulatory pathways. Interruption of this signaling pathway with CD28 antagonists not only results in the suppression of the immune response, but in some cases induces antigen-specific tolerance. However, the CD28/B7 system is increasingly complex due to the identification of multiple receptors and ligands with positive and negative signaling activities. This review summarizes the state of CD28/B7 immunobiology both in vitro and in vivo; summarizes the many experiments that have led to our current understanding of the participants in this complex receptor/ligand system; and illustrates the current models for CD28/B7-mediated T cell and B cell regulation. It is our hope and expectation that this review will provoke additional research that will unravel this important, yet complex, signaling pathway.

INTRODUCTION

One of the fundamental elements of the immune system has been the evolving strategies for exquisite self/nonself discrimination. The T cell receptors (TCR) and B cell receptor (BCR) play a major role in defining the fine specificity of an immune response. However, other mechanisms have been adapted to protect the

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body from untoward autoimmune reactions and to focus the immune response on the infected target tissue. These mechanisms include a unique architectural structure of the lymphoid tissue, the evolution of professional antigen presenting cells (APCs), localized antigen deposition, and homing receptors that bring the relevant immune cells to the inflammatory sites. Another mechanism that has developed as both a backup and amplification system has been the requirement for two distinct signals for T cell and B cell activation (1, 2). One signal originates from the ligation of the T cell receptor (TCR) complex and its co-receptors (e.g. CD4 and CD8). The second signal is dependent on either soluble factors such as IL-2 or the ligation of cell surface molecules that provide essential costimulatory signals complementary to the TCR engagement (3, 4). The costimulatory interactions are necessary for effective lymphocyte activation and also serve to enhance the immune response.

It is now accepted that a major T cell costimulatory pathway involves the CD28 molecule. CD28 interactions with the B7 family of costimulatory ligands are essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell expansion and differentiation. However, as is evident in this review, we have just begun to understand the complexity of the CD28/B7 costimulatory pathway.

THE CD28/CTLA-4 GLYCOPROTEINS

Expression of CD28 and CTLA-4

The CD28 glycoprotein is expressed constitutively on the surface of 80% of human T cells (all $CD4^+$ cells and about 50% of $CD8^+$ cells express CD28) and on virtually 100% of murine T cells (5, 6). CD28 is also highly expressed on developing thymocytes, although its role in T cell development is not well understood (see below) (7, 8). CD28 expression is not static because the levels of CD28 increase on T cells following activation (9). However, ligation of CD28 with either anti-CD28 mAb or B7-1-transfected cells in the presence of PMA or PHA stimulation downregulates CD28 mRNA levels and cell surface expression. There was a concomitant reduction in CD28 signaling as CD28-induced calcium mobilization was significantly reduced (10). CTLA-4, on the other hand, is not constitutively expressed on T cells. Instead, it is upregulated following T cell activation due to positive regulatory elements (11–13; Z Wang, CE Donavan, H He, J Listman, G Guan, T Walunas, JA Bluestone, DL Perkins & PW Finn, unpublished observations). In both human (11) and murine (14) systems, cell surface expression of CTLA-4 peaks 48 h after activation, returning to background levels by 96 h. Thus, the period of time in which CD28 is transiently downregulated and less responsive to signaling is the time period

during which CTLA-4 expression is maximal, suggesting that CTLA-4 may be functionally active at a time when CD28 function is impaired.

The ligation of the CD28 molecule appears particularly important for CTLA-4 upregulation and function. $CD28^-$ human PBLs do not upregulate CTLA-4 mRNA following stimulation by PMA and calcium ionophore (15). Furthermore, anti-CD28 mAbs accelerate the kinetics of CTLA-4 mRNA accumulation among human PBL, such that CTLA-4 mRNA was observed as little as 1 h following activation in the presence of anti-CD28 and at a peak by 6 h (15). Finally, activated $CD28^{-/-}$ murine T cells did not express significant amounts of CTLA-4 at the cell surface unless exogenous IL-2 was added, suggesting that CD28 regulates CTLA-4 expression (14). Linsley and colleagues demonstrated a role for integrins in the regulation of CTLA-4 expression (11, 16). Purified human T cells do not upregulate CTLA-4 following stimulation with anti-TCR mAb alone. The addition of either an immobilized form of soluble ICAM-1, a ligand for LFA-1, or VCAM-1, a ligand for the VLA-4 accessory molecule, resulted in significant expression of CTLA-4 (11, 16). It is, therefore, likely that several signaling pathways play an important role in controlling the expression of CTLA-4.

Finally, one of the more interesting and puzzling aspects of CTLA-4 biology is the discordance between mRNA transcription and cell surface expression. We, and others, have shown that although mRNA levels are increased rapidly following T cell activation, cell surface expression is delayed (15). Activated $CD4^+$ and $CD8^+$ populations of both human (11, 13) and murine (12) origin express CTLA-4 mRNA, as do human and murine Th1 and Th2 T cell clones (13). Detailed studies have shown that CTLA-4 mRNA levels peak approximately 24 h following stimulation with PMA or alloantigen, subsequently decreasing to near background levels by 72 h (11, 15). In fact, in many T cell clones, little, if any, CTLA-4 is expressed following T cell activation even though mRNA is readily detectable in the same cells (R Abe, TL Walunas, C June & JA Bluestone, unpublished observations). In fact, efforts to develop full-length CTLA-4 transfectants and CTLA-4 transgenic mice have to date been unsuccessful. These results suggest that either CTLA-4 expression is post-transcriptionally regulated, or other, as yet unidentified, CTLA-4–associated proteins are needed for transport and cell surface expression.

General Protein Characteristics

Both CD28 and CTLA-4 exist as disulfide-linked homodimeric glycoproteins (4, 5, 11, 17). However, several groups have suggested that these molecules can also exist as monomeric proteins (15, 18). In our studies, murine CTLA-4 was found to exist on the surface of activated T cells as both a disulfide-linked dimer (with the monomer migrating between 33 and 37 kD) and a non–disulfide-linked

monomer (14). Individual anti-murine CTLA-4 mAbs preferentially precipitated monomeric vs dimeric forms of the glycoprotein (18). The two molecules are likely to exist in multiple isoforms on the cell surface (18). Discrepancies between the various studies may be the result of variations in the serological reagents. Both CD28 and CTLA-4 have an unpaired cysteine residue at a position just proximal to the transmembrane domain (19). This site is believed to be the site of dimerization for both CD28 and CTLA-4 monomers and could potentially be involved in the formation of heterodimers between CTLA-4 and CD28 glycoproteins. Although genetic and biochemical studies do not support this possibility, anti-human CTLA-4 mAb specifically immunoprecipitated CTLA-4 from COS cells cotransfected with human CD28 and CTLA-4 (11). In addition, there was no evidence for depletion of CTLA-4 following preclearing of membrane extracts of activated human T cells with anti-CD28 mAb (15). Finally, CTLA-4 precipitated from activated $CD28^-$-deficient ($CD28^{-/-}$) murine T cells was identical to that observed from $CD28^+$ T cells (14). Thus, it appears that heterodimerization of CD28 with CTLA-4 is not required for CTLA-4 expression on the cell surface, and vice versa, although low levels of functional heterodimer expressed on activated T cells cannot be completely ruled out.

Regulation of T Cell Activation by CD28 and CTLA-4

THE ROLE OF CD28 IN THE REGULATION OF CELL CYCLE PROGRESSION AND APOPTOSIS The proliferative responses of T cells isolated from $CD28^{-/-}$ mice or $CD28^+$ mice stimulated in the presence of CD28 antagonists are substantially reduced (21). However, recent studies have shown that CD28/B7-mediated signaling does not affect initial T cell proliferation (24–48 h) but attenuates the late proliferative responses (20, 21; JA Bluestone, unpublished observations). This is manifested as increased T cell death (apoptosis) late in culture that cannot be fully reversed by the addition of IL-2 or other survival cytokines. Thus, although CD28/B7 interactions clearly can facilitate the initiation of T cell responses, the major role of CD28 signaling may be to prevent apoptosis and help sustain proliferation. In this regard, a recent study has focused on the role of CD28 in the regulation of two genes believed to be involved in the prevention of cell death, bcl-2 and bcl-x. Both bcl-2 and the high molecular weight form of *bcl*-x, *bcl*-x_L, protect lymphocytes from apoptosis (22–24). Boise et al showed that while CD28 costimulation does not lead to any changes in the levels of bcl-2 found in activated T cells, that it does upregulate bcl-x_L and that this upregulation correlates with protection from cell death by a Fas dependent mechanism (25).

TH1/TH2 SUBSET DIFFERENTIATION CD28 engagement has been shown to enhance the production of various cytokines, including IL-1, IL-2, IL-4, IL-5,

TNF, and IFN-γ (5, 26, 27). However, recent studies in vitro and in vivo support a fundamental role for CD28 in the early development and differentiation of both Th1 and Th2 T cell subsets (28–30; LMC Webb, M Feldmann, personal communication). In the absence of CD28 signaling, naive T cells are biased toward a Th1 phenotype. Seder et al demonstrated that no IL-4 was produced when CD28/B7 interactions were blocked with hCTLA4Ig (28). In this system, the lack of IL-2 production was the underlying cause for the effect, since the addition of exogenous IL-2 overcame the defect in IL-4 production. In additional studies, differentiation of human Th1/Th2 subsets was shown to be dependent on CD28 ligation. Purified naive human T cells stimulated with anti-CD3 in the absence of CD28 costimulation produced only IL-2 and IFN-γ, whereas the addition of anti-CD28 mAbs induced both IL-4 and IL-5 (29; LMC Webb, M Feldmann, personal communication). The requirement for CD28 could not be overcome in these cultures by the addition of exogenous IL-4, suggesting that CD28 ligation may regulate other factors involved in T cell responsiveness to IL-4.

The importance of CD28 costimulation in the differentiation of Th2 cells is supported by several studies in vivo. The $CD28^{-/-}$ mice have a reduced Th2-dependent antibody response to VSV, while the Th1-dependent DTH response to LCMV remains intact (31). In addition, $CD28^{-/-}$ mice can reject allografts (DJ Lenschow, J Green, Y Zeng, CB Thompson, & JA Bluestone, unpublished data). The murine CTLA4Ig transgenic mice display a similar phenotype, with the most dramatic defect being a hyporesponsive B cell compartment (32, 33). T cells isolated from CTLA4Ig transgenic mice produce significantly decreased amounts of IL-4 and increased amounts of IFN-γ in response to primary immunization, and the B cells demonstrated impaired humoral responses to multiple antigens as compared to wild-type animals (32, 33). Furthermore, in the murine model of leishmaniasis the administration of hCTLA4Ig at the time of inoculation resulted in protection in the susceptible, Th2-type BALB/c mouse strain, but it had no effect in the resistant, Th1-type C57BL/6 mouse strain (34). Finally, hCTLA4Ig treatment blocked the induction in vivo of an IL-4 response to a nematode parasite, *Heligmosomoides polygyrus*. Mice treated with hCTLA4Ig at the time of infection generated significantly reduced numbers of IL-4 secreting cells, decreased levels of serum IgE, and reduced blood eosinophil counts (35). These results are most consistent with a model in which CD28-mediated signaling directly regulates Th1/Th2 differentiation.

CTLA-4 FUNCTION Although CD28 is clearly a costimulatory molecule, the function of CTLA-4 remains controversial. Early studies predicted that CD28 and CTLA-4 might play similar roles in the regulation of T cell responses, due to the amino acid sequence homology and ligand binding specificity. The first studies examining the role of CTLA-4 in proliferative responses in vitro

suggested that CTLA-4, like CD28, was indeed a costimulatory molecule. Immobilized anti-human CTLA-4 mAbs in the presence of TCR stimulation augment proliferation, although to a lesser degree than does anti-CD28-mediated costimulation. When both mAbs were present together with the anti-TCR stimulus, synergistic proliferation was observed (11). However, additional functional studies in both the murine and human systems suggested a different role for CTLA-4. Whereas anti-murine CTLA-4 mAb augmented proliferation of purified T cells, monovalent Fab fragments of the anti-CTLA-4 mAb, which do not cross-link CTLA-4, also augmented proliferation (14). Thus, CTLA-4 cross-linking may not transduce a positive signal, but rather the anti-CTLA-4 mAbs may block a negative signal by interrupting the interaction of CTLA-4 with its counterreceptors. In fact, whole anti-CTLA-4 mAbs inhibited anti-CD3-mediated T cell activation in the presence of optimal CD28 costimulation (14, 36). Recent studies in vivo also support the role for CTLA-4 as a downregulatory molecule in T cell activation. Kearney et al have described an adoptive transfer model in which antigen-specific transgenic T cells can be monitored for their expansion following exposure to antigen (37, 38). In these studies, animals treated with either anti-CTLA-4 mAb or Fab fragments of the anti-CTLA-4 mAb showed augmented antigen-mediated clonal expansion. There was also a delay in the decay in T cell numbers over time, suggesting that the anti-CTLA-4 mAb blocked programmed cell death in vivo (38). Thus, during T cell activation, CTLA-4 is upregulated, and through interactions with its ligands may facilitate or promote downregulation of the immune response. In this regard, Gribben et al have shown that ligation of CTLA-4 may mediate apoptosis (39). When preactivated human T cells were restimulated with antigen in the presence of an anti-CTLA-4 mAb, the proliferation was decreased, and the majority of T cells underwent programmed cell death. Induction of apoptosis in the presence of anti-CTLA-4 mAb could be blocked by coculturing the cells with either anti-CD28 mAb or exogenous IL-2, consistent with recent evidence that CD28 signaling upregulates the cell survival factor bcl-x_L (25). These results suggest that CTLA-4 may function through an association with an additional cell surface molecule.

THE B7-1 (B7/CD80) AND B7-2 (B70/CD86) GLYCOPROTEINS

B7-1, a B cell activation molecule first described in 1981 by Yokochi et al (40), was the first ligand to be identified for CD28, and later for CTLA-4 (41). In addition to its expression on activated B cells, B7-1 was also detected on a variety of APCs including dendritic cells, Langerhans cells, activated

monocytes, activated T cells, and a variety of tumor lines (Table 1). Both the human and murine B7-1 genes were cloned by Freeman et al (42, 43) and shown to be members of the immunoglobulin superfamily. The functional importance of the B7-1 molecule has been demonstrated in a number of studies of T cell activation. Both anti-CD3 and PMA-induced T cell proliferation was augmented by the addition of B7-1 transfectants. The proliferation was enhanced in a CD28-dependent fashion since T cell activation was blocked by anti-CD28 mAbs (44, 45). Furthermore, the potent costimulatory role of B7-1 has been demonstrated in vivo in transgenic mice in which B7-1 was ectopically expressed on the cells of the islets of Langerhans (46–48).

Despite the apparent ability of B7-1 to provide sufficient costimulation when expressed on transfectants or transgenic mice, it has been very difficult to demonstrate its function on normal antigen-presenting cells in mice. Anti-B7-1 mAbs minimally inhibit a primary mixed lymphocyte response, while hCTLA4Ig inhibits the response by as much as 80% (49). Additionally, the staining of either LPS-activated B cells or whole spleen with hCTLA4Ig was not inhibited by anti-B7-1 mAbs, suggesting that an additional CTLA-4 ligand existed (49–52). Finally, B7-1$^{-/-}$ mice are capable of mounting an immune response to nominal antigens and antigen-presenting cells isolated from these mice could be stained with labeled hCTLA4Ig (53). These observations led to the identification and eventual cloning of a second B7 family member, B7-2 (54, 55).

The human B7-2 gene was cloned by Freeman et al (55) and Azuma et al (54). The overall structure of B7-2 was found to be very similar to B7-1 with an extracellular domain containing two Ig-like domains, a transmembrane domain, followed by a cytoplasmic tail (54, 55). The cytoplasmic tail contains three potential sites for protein kinase C phosphorylation, indicating a potential signaling role for this molecule. In fact, the B7-2 molecule is phosphorylated following B cell activation (L Lanier, unpublished observations). The murine homolog of B7-2 has also been cloned (56, 57). These genes share only 25% amino acid homology with their B7-1 counterparts. However, several areas of homology, centered around sequences required for the formation of the Ig domains and potential CD28/CTLA-4 binding sites, have been reported (56).

Similar to B7-1 transfectants, B7-2 transfectants augment T cell proliferation and IL-2 production to suboptimal stimulation with anti-CD3 or PMA. This costimulation was inhibited by either hCTLA4Ig or anti-CD28 Fab, but not by anti-B7-1 mAbs, demonstrating therefore that B7-2 binds to both CD28 and CTLA-4 (54–56). While anti-B7-1 mAbs have been inefficient in their ability to block primary allogeneic MLRs, in most cases, the anti-B7-2 mAbs inhibited the responses to levels similar to hCTLA4Ig (58–60). A combination of both

Table 1 Cellular distribution of human and mouse B7-1 and B7-2

Cell type	Stimulation Conditions	hB7-1	hB7-2	mB7-1	mB7-2	References
B cell	Resting	none	none	none	low	(42, 49, 54, 58)
	Cultured	-[a]	↑	-	↑	(54, 58)
	LPS	↑	↑	↑	↑	(49, 54, 58, 60)
	Ig cross-linking[c]	↑	↑	-	↑	(43, 58, 59)
	Anti-CD40	↑	↑	↑	↑	(54)
	IL-2	↑	NR[b]	NR	↑	(71, 72)
	IL-4	↑	↑	↑	↑	(71, 72)
	IL-5	NR	NR	NR	↑	(58)
	IFN-γ	NR	NR	NR	↑	(72)
T cells	Resting	none	none	none	low	(54, 58, 63)
	Anti-CD3	↑	↑	↑	↑	(54, 58, 112)
T cell clones	Resting	-	-	-	-	(54, 112, 114)
	Anti-CD3	↑	↑	↑	↓	(54, 112, 114)
Peripheral blood monocytes	Resting	low	high	none	NR	(42, 54)
	IFN-γ	↑	↑	↑	NR	(42, 54, 68, 73)
	GM-CSF	↑	↑	NR	NR	(68)
	FcR cross-linking	↓	↓	NR	NR	(68)
Peritoneal macrophages[d]	Freshly isolated	NR	NR	low	low	(58)
	LPS	NR	NR	↑	↑	(58)
	IFN-γ	NR	NR	↓	↑	(58)
	IFN-γ + IL-10	NR	NT	↓[e]	↓[e]	(75, 115)
Peripheral blood dendritic cell	Resting	low	high	low	low	(54)
	IL-10	-	↓	NR	NR	[f]
Splenic dendritic cells	Freshly isolated	NR	NR	low	low	(63)
	Cultured	NR	NR	↑	↑	(49, 63, 116)
	LPS	NR	NR	NR	-	(63)
Langerhans cells	Freshly isolated	none[e]	low[e]	none	low	(63, 117)
	Cultured	↑	↑	↑	↑	(63, 117)

[a]No change
[b]NR = not reported
[c]The cross-linking of surface Ig with antigen or anti-Ig did not induce B7-1. B7-1 was induced when surface Ig was highly cross-linked by Ig-dextran.
[d]Peritonal macrophages were thioglycollate induced.
[e]These cells were stained with CTLA4Ig and therefore B7-1 and B7-2 cannot be differentiated.
[f]C Buelens, F Willems, A Delvaux, G Pierard, J-P Delville, T Velu & M Goldman, personal communication.

anti-B7-1 and anti-B7-2 mAbs were the most effective at inhibiting the MLR (61, 62). The role for both molecules in primary responses is further supported by the finding that a combination of anti-B7-1 and anti-B7-2 antibodies can induce anergy (61).

Resting B cells express no detectable B7-1 and very low levels of B7-2, while both B7-1 and B7-2 are upregulated following B cell activation with agents such as LPS, Con A, or cAMP (42, 49, 54, 58) (Table 1). However, dramatic differences exist in the kinetics and the signals that control B7-1 and B7-2 expression. The induction of B7-2 occurs within 6 h of stimulation, with maximal levels of expression achieved between 18 and 24 h (49, 58). In contrast, B7-1 expression is not detected until 24 h post stimulation and does not reach maximal levels until 48 to 72 h later (49, 58). Furthermore, activated B cells and dendritic cells expressed quantitatively higher levels of B7-2 than of B7-1, since hCTLA4Ig staining was almost completely inhibited by anti-B7-2 mAbs, while anti-B7-1 mAbs had little effect (49, 58, 63). Differences in expression were observed not only on B cells, but also on T cells. Freshly isolated human and murine T cells express low levels of B7-2 but not of B7-1 (54, 58, 63). Both B7-1 and B7-2 expression were upregulated following activation with anti-CD3 (54, 58). The regulation of B7-1 and B7-2 expression is controlled by cell-cell interactions and cytokines. Signals delivered through the cytoplasmic tail of the MHC class II molecules induced B7-1 expression on B cells (65, 66). Cells expressing tailless class II were profoundly deficient in their antigen presenting capacity, which correlated with a lack of B7-1 induction. Treatment of these cells with dibutyryl-cAMP restored their ability to present antigens by inducing B7-1 expression. The cross-linking of surface Ig also regulated B7-1 and B7-2 expression. B7-2 was rapidly induced on the B cell surface following cross-linking with anti-Ig or antigen (58, 59).

Following the engagement of antigen, the ability of surface Ig to rapidly induce functional B7-2 transforms a resting B cell into a fully competent antigen presenting cell (67). In contrast, BCR cross-linking with either anti-Ig or antigen did not induce detectable levels of B7-1, although IgD-coated beads resulted in some increased expression of B7-1 (43, 58). Together these results suggest that the degree of cross-linking determines whether or not B7-1 is upregulated. Interestingly, engagement of the Fc receptor downregulates both B7-1 and B7-2 on monocytes that have been activated with either IFNγ or GM-CSF (68). Thus, the engagement of distinct cell surface molecules may have antagonistic effects and may, in part, explain the inhibitory effect of Fc receptor cross-linking on B cell function. Finally, the CD40/CD40L pathway plays an important role in controlling B7-1 and B7-2 expression. Both B7-1 and B7-2 were induced by signaling through CD40, either through anti-CD40 mAbs or

activated T cells that expressed the CD40 ligand (69, 70). Thus, a variety of cell surface glycoproteins on B cells can regulate B7-1 and B7-2 expression and APC function.

An additional level of control of B7-1 and B7-2 expression results from the influence of cytokines. A number of cytokines have been shown to differentially regulate B7-1 and B7-2 expression. IL-4 is one of the most potent inducers of B7-2 and, to a lesser extent, B7-1 on B cells (71, 72). Incubation of small resting B cells with IL-4 upregulates B7-2 expression within 6 h with maximal induction occurring by 24 h (72). IFNγ increases the expression of B7-2 on B cells, peritoneal macrophages, and peripheral blood monocytes (58, 68, 72). IFNγ also increases the expression of B7-1 on peripheral blood monocytes but, surprisingly, downregulates expression of B7-1 on peritoneal macrophages (58, 73). IL-10 blocks both B7-1 and B7-2 upregulation on peritoneal macrophages and downregulates B7-2, but not B7-1, on human dendritic cells (74, 75; C Buelens, F Willems, A Delvaux, G Pierard, J-P Delville, T Velu & M Goldman, personal communication). These results suggest that the immunosuppressive properties of IL-10 may, in part, be a result of its regulation of CD28/CTLA-4 ligands. Thus, the differences in the ability of the respective cytokines to regulate the levels and temporal expression of B7-1 and B7-2 both qualitatively and quantitatively may result in distinct effects during an immune response.

ROLE OF CD28/B7 IN T CELL–B CELL INTERACTIONS

In order for B cells to enter the cell cycle, produce Ig, and undergo Ig class switching or somatic hypermutation, it is necessary for them to receive the appropriate T cell help (76). Recent work has demonstrated that both CD28/B7 and CD40/CD40L signaling pathways play a critical role in B cell responses. However, the interactions of the CD28/B7 and CD40L/CD40 pathways are not well understood. Early studies showed that signaling through the CD28 receptor can increase the surface expression of CD40L (77, 78; SJ Klaus, J Rosser, EA Clark, personal communication). Furthermore, the activation of the T cells by cross-linking CD28 with either antibody or with B7-1 transfectants enhanced the production of IgG and IgM by B cells in vitro as compared to anti-CD3-stimulated T cells alone (77). However, signaling through the TCR alone was sufficient for CD40L upregulation, and hCTLA4Ig did not block CD40L expression or the ability of these antigen-activated T cells to provide B cell help (70, 78). These results are consistent with the idea that CD28 ligation is not required for CD40L induction, but, as reported, CD28 ligation stabilizes CD40L mRNA, allowing for more rapid translation and transport of $CD40_L$ the T cell surface, and therefore results in increased B cell responses (78; SJ Klaus, J Rosser, EA Clark, personal communication).

The interrelationship between the CD28/B7 and CD40L/CD40 pathways also plays an important role in the ability of B cells to present antigen. While resting B cells express little if any B7-1 or B7-2, CD40 ligation induces their expression. B cells activated with anti-CD40 mAbs stimulated an allogeneic MLR more efficiently, and this stimulation was blocked by hCTLA4Ig. Further investigation revealed that CD40 cross-linking by mAbs, or the interaction of CD40 with its ligand CD40L, upregulated B7-1 and B7-2 (69). Incubation of resting B cells with activated T cells is another potent means of inducing B7-1 and B7-2 (70). In this system, B7-1 upregulation was inhibited by either anti-CD40 or by anti-CD40L mAbs consistent with the notion that CD40L/CD40 regulates B7-1 expression (70). On the other hand, the CD40L antagonist did not completely inhibit the ability of activated T cells to upregulate B7-2 (70). Therefore, other signals mediated by class II engagement or cytokines are involved in B7-2 regulation by activated T cells.

A TEMPORAL MODEL FOR THE REGULATION OF CD28/B7 FAMILY MEMBERS

Based on the studies summarized above, it is clear that the interactions of the CD28/B7 family members are highly regulated and quite complex. One model for the regulation of immune responses by CD28/B7 family is based on the kinetics of expression of the various CD28/B7 ligands and the ability of the B7-1, B7-2, CD28, and CTLA-4 molecules to cross-bind. First, B7-2 is rapidly induced on B cells through the engagement of surface Ig with antigen, cytokines produced by previously activated T cells, or CD40 cross-linking by activated T cells. Next, antigen-specific naive or activated $CD28^+$ T cells can interact with the $B7\text{-}2^+$ B cells expressing a complete MHC/antigen complex. The simultaneous engagement of the TCR and the CD28 molecules results in the activation of the T cell and prevents the induction of anergy. Signals generated through the T cell receptor induce the expression of CD40L on naive cells, which is further stabilized by CD28 signals. The upregulation of CD40L then allows the T cell to provide the necessary B cell help for the production of antibodies. Concurrently, APCs upregulate B7-1 levels, via signaling through MHC class II, CD40, and perhaps lymphokines secreted by the activated T cells.

One to three days after this cellular interaction occurs, CTLA-4 is upregulated on T cells. Several interactions are possible at this time since the B cells express both B7-1 and B7-2, while the T cells express both CD28 and CTLA-4. Since CTLA-4 has a higher affinity for B7-1 and B7-2 than does CD28, these costimulatory ligands may preferentially interact with CTLA-4 on the activated T cells to downregulate the immune response either by directly inducing

apoptosis or by competing with CD28 for its ligands and preventing the upregulation of protective factors such as bcl-x_L. Thus, under normal circumstances, the CD28/B7 system will serve to both upregulate and downregulate immune responses. Any disturbance in the CD28/B7 system may disregulate the immune response and affect the development and progression of the immune response.

IN VIVO REGULATION OF IMMUNE RESPONSE BY MANIPULATION OF THE CD28/B7 PATHWAY

Activation of Immune Responses

Both B7-1 and B7-2 costimulate T cells through their interaction with CD28. The expression of one of these ligands on a cell that lacks costimulatory molecules could convert a nonfunctional APC into a functional one. This may have important implications in upregulation of immune responses. In fact, CD28/B7-mediated costimulation has provided a new approach to cancer therapy, because the inability of some tumors to induce immune responses has, in some cases, been correlated with a deficiency in providing costimulatory signals. The introduction of B7-1 or B7-2 into tumor cells in several models enhances the anti-tumor response (79–86). In fact, in some studies, the immunization of mice with the B7-1-expressing tumor protected the animal from further challenge with nontransfected parental tumors (80–86). However, the transfection of B7-1 into tumor cells has not been uniformly successful in inducing tumor immunity. In one instance, a potent immune response was induced and the B7-1 transfected tumors were rejected, but these same tumors could not protect from further challenge with the parental tumor (79). Furthermore, the lack of an anti-tumor response did not always depend on a deficient costimulatory response. Several relatively nonimmunogenic tumors did not induce an immune response, even when cotransfected with both B7-1 and B7-2 (83, 85, 87). These results suggest that certain tumors may be lacking additional cell surface molecules needed for complete T cell activation. These additional factors may include the tumor antigen itself, MHC molecules, or other cell surface costimulators. In support of this possibility, cotransfection of one of the nonimmunogenic tumors with both B7-1 and ICAM-1 resulted in its immune recognition and rejection (86).

Studies have begun to compare the relative costimulatory activity of B7-1 and B7-2. The majority of studies in vitro and some tumor studies in vivo (87) suggested that B7-2 was as effective a costimulatory molecule as B7-1, recent experiments in which either B7-1 or B7-2 were transfected into tumors have suggested that B7-1 is a more potent costimulator in some tumor models (CJ Bartels, JC Yang, personal communication; T Gajewski, personal

communication). In another study, in which the animals were immunized with irradiated tumor cells, only the B7-1-transfected tumors were able to generate tumor-specific CTLs and subsequently to protect against tumor challenge (T Gajewski, personal communication). Interestingly, in both models, the combined expression of B7-1 and B7-2 on the tumor cells was less effective in stimulating an anti-tumor response than was expression of B7-1 alone. This may reflect a competition between B7-1 and B7-2 for binding to CD28. Alternatively, the addition of B7-2 to the transfectant may hyperstimulate the immune response to shut down T cells either directly, perhaps through its interaction with CTLA-4, or by increasing the potency of the T cell signals to promote Th2 responses that inhibit tumor immunity (see below).

Suppression of Immune Responses

In vitro studies have demonstrated that the CD28/B7 pathway is critical in T cell activation. The interruption of this pathway leads to an inhibition of T cell proliferation and, under some circumstances, induces either antigen-specific hyporesponsiveness or anergy (88, 89). Therefore, targeting this pathway in vivo may represent a novel method of immunosuppression in which only the antigen-specific T cells would be tolerized. To examine this possibility, several investigators have utilized hCTLA4Ig to interrupt the CD28 signaling pathway in several models including transplantation, autoimmune disease, antibody responses, and parasite challenge.

TRANSPLANTATION The importance of CD28/B7 interactions in vivo was first established in the transplant setting. CTLA4Ig treatment effectively prolonged graft survival and, in many cases, induced donor-specific tolerance. As an example, in a xenogeneic islet transplant model, diabetic mice transplanted with human islets and treated with CTLA4Ig exhibited long-term survival of the xenogeneic islets (90). Furthermore, this short treatment induced donor-specific tolerance. hCTLA4Ig-treated mice that were retransplanted with either donor or third party islets rejected only the third party islets. Human CTLA4Ig prolonged graft survival in both allogeneic rat cardiac and murine islet transplant models, but eventually all of the grafts were rejected (62, 91). Human CTLA4Ig treatment also reduced the lethality of allogeneic graft-vs-host disease (GVHD) across an MHC barrier but did not alleviate all of the symptoms of GVHD (92, 93). These results suggest that allogeneic transplantation may be more difficult to manipulate than the xenogeneic systems. However, recent efforts to augment the effectiveness of hCTLA4Ig have been successful. Pearson et al found that hCTLA4Ig treatment could induce donor-specific tolerance in a rat cardiac allograft model in certain strain combinations (94). In the studies performed by Turka and colleagues, changes in the dose regimen or the addition

of donor-specific transfusions led to donor-specific tolerance (95, 96). Finally, the addition of other immunosuppressive drugs maximizes the hCTLA4Ig-mediated suppression. The blockade of CD28/B7 and LFA-1/ICAM-1 interactions was more effective than hCTLA4Ig alone at preventing GVH disease-induced lethality (BR Blazar, PA Taylor, A Panoskaltsis-Mortari, GS Gray, DA Vallera, personal communication). Treatment of mice with hCTLA4Ig and suboptimal doses of cyclosporin A (CsA) at the time of transplant resulted in graft survival beyond that of hCTLA4Ig alone and in some cases resulted in indefinite graft survival (97; Y Zeng, JA Bluestone, unpublished observations). The ability of hCTLA4Ig and CsA more effectively to block transplant rejection has important clinical implications because current transplant therapies utilize CsA. All in all, these studies demonstrated the effectiveness of interrupting the CD28/B7 signaling pathway in promoting transplant tolerance. These studies also suggest that the timing of the treatment or the strength of TCR signal may be important in determining the efficacy of hCTLA4Ig immunosuppression.

HUMORAL RESPONSES One immune response that has been profoundly inhibited by hCTLA4Ig therapy is the humoral immune response. Primary antibody responses to soluble proteins such as KLH or cell-bound antigens such as sheep red blood cells were inhibited by hCTLA4Ig in a dose-dependent fashion (98). Treatment with hCTLA4Ig could be delayed as long as 3 days following antigen priming and still suppress in vivo antibody production. Human CTLA4Ig therapy during the primary response also reduced secondary and tertiary responses to the nominal antigen. The amount of reduction correlated with the concentration of hCTLA4Ig used in the initial treatment, suggesting that the immunosuppression lasted only for the duration of circulating serum levels (98). However, anti-B7-2 mAb treatment not only blocked antibody production to antigenic challenge but also suppressed somatic hypermutation and had long-term effects on B cell memory responses (99). Therefore, the use of the more potent CD28 antagonists may be the most promising treatment in vivo.

Further evidence that CD28/B7 signaling plays an important role in antibody responses has come from the examination of $CD28^{-/-}$ mice. Antibody production in these mice was severely impaired. Basal immunoglobulin levels are only 20% of that observed in normal mice. Furthermore, the relative proportions of the different IgG subclasses were altered. The serum of these animals contains dramatically decreased levels of IgG1 and IgG2b, while the levels of IgG2a were increased as compared to normal mice. B cell responsiveness in the $CD28^{-/-}$ mice appeared to be intact since normal levels of anti-VSV Abs of the IgM class (a T-independent response) were produced in response to a primary challenge (31). However, the T cell help necessary for antibody class switching

following a second challenge was not intact in these mice. Transgenic mice expressing soluble murine CTLA4Ig displayed a phenotype very similar to that of $CD28^{-/-}$ mice. T-independent antibody responses were intact, but immune responses to T-dependent antigens were severely impaired (32, 33). Primary responses to DNP-KLH were eliminated. Only after two and three stimulations could reduced levels of antibodies be detected, and these were of the IgM, not the IgG isotype. This deficiency in T cell help was also evident by a lack of germinal center formation, isotype switching, and somatic hypermutation (32, 33) similar to what had been observed in the anti-B7-2–treated animals (99). Therefore, blockade of the CD28/B7 interactions, either from the CD28 side or the B7 side, results in a profound defect in the ability of B cells to respond to antigen in vivo.

AUTOIMMUNE DISEASES CTLA4Ig therapy has also had significant effects on the clinical course of several autoimmune diseases. We have shown that blockade of the CD28/B7 pathway has profound effects on the development of diabetes in the NOD mouse model. In these studies, hCTLA4Ig treatment of young NOD mice inhibited the onset of diabetes that occurred 6 to 18 weeks after treatment was stopped and the protein had been cleared from the serum (100). In a relapsing model of experimental autoimmune encephalomyelitis (R-EAE), hCTLA4Ig treatment during antigen priming blocked the development of clinical disease (101). This was also true in a T cell adoptive transfer model of R-EAE (102). In the murine model for systemic lupus erythematosus (SLE), NZB/NZW F_1 mice (B/W) spontaneously develop a lupus-like autoimmune disease characterized by the production of autoantibodies to self-molecules such as dsDNA (103). The treatment of these mice with murine CTLA4Ig prior to the detection of autoantibodies significantly inhibited autoantibody production and disease progression, even after cessation of treatment (104). Thus, blockade of CD28/B7 signaling can control early events involved in the induction of several different autoimmune diseases. However, most autoimmune diseases are diagnosed after initial responses to the autoantigen(s). Therefore, it was important to examine the effectiveness of these therapies on established disease. Treatment of the lupus-prone mice with murine CTLA4Ig during the late stages of the disease effectively intervened in disease progression and prevented the production of further autoantibodies (103). In addition, NOD mice treated with hCTLA4Ig after the onset of insulitis also had a reduced incidence of diabetes (100). Finally, relapses of clinical disease in R-EAE were inhibited by F(ab) fragments of anti-B7-1 mAbs when treatment was initiated after the resolution of the acute phase of disease (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, JA Bluestone, unpublished observations). Together, these data demonstrate an important role for CD28/B7 signaling in both the initiation and propagation of several autoimmune diseases.

DIFFERENTIAL EFFECTS OF B7-1 AND B7-2 LIGATION

It is becoming increasing clear that the B7-1 and B7-2 molecules may differentially control the immune response as a consequence of one or more of the distinctive properties of these costimulatory ligands. For instance, the expression of B7-1 and B7-2 varies on B cells, T cells, macrophages, and dendritic cells depending on the activation state of the cells (Table 1). An additional level of complexity involves the ability of cytokines to either induce or suppress the expression of these costimulatory ligands. Therefore, either B7-1 or B7-2 may dominate during different stages of the immune response. Furthermore, there are increasing data to suggest that these two molecules do not bind the CD28 and CTLA-4 molecules similarly (105). Both B7-1 and B7-2 bind to hCTLA4Ig with a 20–100-fold higher avidity than they do to CD28Ig. In addition, Linsley and colleagues showed that B7-1 has a slightly higher avidity for CD28 and CTLA-4 than does B7-2. More significantly, B7-2 dissociates more rapidly from hCTLA4Ig than does B7-1, and hCTLA4Ig was less effective at inhibiting B7-2–dependent responses (105). Finally, the fine specificity of the B7-1 and B7-2 interaction with hCTLA4Ig is different. Previous studies have shown that the region of CTLA-4 and CD28 that is the site of interaction with both B7-1 and B7-2 is a transmembrane proximal hexapeptide, MYPPPY, found in the region of greatest sequence homology between CD28 and CTLA-4. A hCTLA4Ig construct containing a single amino acid mutation in the MYPPPY motif showed reduced but still significant binding to B7-1. However, binding to B7-2 was completely abolished. Thus, amino acids within the CDR1 and CDR3 regions of the CTLA-4 molecule are responsible for the enhanced avidity of CTLA-4 for B7-1 (106). However, the precise interaction sites for B7-1 and B7-2 with CD28 and CTLA-4 may be distinct (105).

Recent studies, in vitro and in vivo, suggest that these differences may directly effect CD28-mediated costimulation. Kuchroo et al demonstrated that T cells from a myelin basic protein-specific TCR transgenic mouse secreted predominantly IFN-γ when cultured in the presence of anti-B7-2 mAbs, while cultures carried out in the presence of anti-B7-1 mAbs led to increased IL-4 secretion (107). Studies by Freeman et al also support an important role for B7-2 in the signaling of IL-4 production (108). While transfectants of B7-1 or B7-2 induced similar levels of IL-2 and IFN-γ, only B7-2 transfectants were able to induce IL-4. Continuous restimulation by the B7-2 transfectants resulted in the induction of greater amounts of IL-4 (108). Together, these results suggest that B7-1 and B7-2 may directly control Th1 vs Th2 development, respectively. In vivo studies also suggest that the B7-1 and B7-2 costimulatory ligands play distinct roles during the initiation and propagation of an immune response. Kuchroo and colleagues found that treatment with anti-B7-1 mAbs

during R-EAE induction protected mice from disease, while anti-B7-2 mAbs exacerbated disease severity (107). In this model, the investigators correlated the presence of a Th2 response with the protective therapy and suggested that the anti-B7-2 mAb therapy skewed the T cell response toward a Th1 phenotype. However, a number of other studies in vitro and in vivo have shown that B7-1 and B7-2 regulate both Th1- and Th2-mediated responses. First, two studies have shown that both B7-1 and B7-2 transfectants can provide costimulatory signals for both Th1 and Th2 lymphokine production (109, 110). Second, the primary immune response appears to be most dependent on B7-2 since B7-$1^{-/-}$ mice have relatively normal Th1- and Th2-dependent responses, while B7-$2^{-/-}$ mice are severely compromised (A Sharpe, personal communication). Furthermore, in an allogeneic islet transplant model, anti-B7-2 but not anti-B7-1 mAbs prevented graft rejection. The combination of the two mAbs was most effective in prolonging allograft survival (62). Since graft rejection has been suggested to be Th1-mediated, B7-1 rather than B7-2 would have been expected to be dominant in this setting. Furthermore, the treatment of mice with anti-B7-2 mAbs during priming in vivo with antigen (a Th2-dependent immune response) inhibited the development of antibody responses (52, 99). Anti-B7-2 mAb treatment was also able to block disease progression in the NOD model for autoimmune diabetes (100). Thus, it appeared as if B7-2 played a dominant role as the costimulatory ligand for CD28 in transplantation, humoral responses, and initiation of autoimmune disease.

More recent studies have shown that B7-1 can regulate immune responses, especially following initial antigen exposure. Treatment of NOD mice with anti-B7-1 mAbs accelerated the disease course and exacerbated the inflammatory response in female mice treated at 2 to 4 weeks of age (100). Furthermore, anti-B7-1 treatment of normally resistant male mice developed disease at a rate and frequency similar to that observed in untreated female NOD mice (100). A similar exacerbation of disease was observed with anti-B7-1 mAbs in a murine R-EAE model (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, & JA Bluestone, unpublished observations). Treatment initiated after resolution of the acute phase of disease resulted in an increased rate of onset, frequency, and severity of the relapses. However, treatment with non-cross-linking F(ab) fragments of the anti-B7-1 mAbs immediately after the resolution of the acute phase of disease blocked both clinical relapses and epitope spreading in R-EAE. Two points can be made from these studies. First, the exacerbation caused by the intact anti-B7-1 mAb may be due to its direct signaling of either the APCs or activated T cells. This possibility has important implications for all the in vivo studies that utilize antibody therapy. Second, there appear to be distinct differences on the outcome of the mAb therapy depending on the temporal

administration of the antibodies. The B7-2 molecule appears to play a critical role prior to antigen exposure (transplant setting and humoral responses), whereas B7-1 plays an important role in the control of the immune response after antigen exposure. One explanation for these differences may be our recent observation that B7-1 expression is preferentially upregulated during the acute phase of an autoimmune response (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, & JA Bluestone, unpublished observations). In fact, many of the in vivo effects of the CD28 antagonists can be explained by the timing of antibody therapy. The treatment of BALB/c mice with hCTLA4Ig during infection with *L. major* protected the normally susceptible strain from disease, by decreasing the production of IL-4, while the mice were no longer protected from disease by hCTLA4Ig if treatment was delayed by more than one week post-infection (34). The efficiency of hCTLA4Ig blockade of antibody responses was also decreased as treatment was delayed (98). Finally, in an allogeneic cardiac transplant model, delaying treatment with hCTLA4Ig by 2 to 3 days post-transplant resulted in long-term survival characterized by an inhibition of Th1 but not Th2 cytokines (96). Thus, a variety of factors, including the nature of the APC expressing the B7 molecules, the affinity of the B7 molecules for CD28 or CTLA-4, and the level of B7-1 and B7-2 expression, may determine the relative roles played by each ligand during an immune response. Furthermore, the timing of treatment with CD28 antagonists can influence the Th1 and Th2 subset development.

STRENGTH OF SIGNAL MODEL

Based on all of these observations, we propose a model of costimulation that integrates many of the observations related to TCR ligation, CD28 costimulation, and Th1/Th2 biology (Figure 1). The "strength-of-signal" hypothesis suggests that the intensity of T cell signaling not only determines the potential to initiate a response but can dramatically affect the balance of Th1/Th2 subsets. We speculate that CD28 costimulation can have distinct effects on the immune response depending on antigen dose, APC function, cytokine milieu, and level of costimulation. Under conditions of low antigen density, CD28 ligation is essential since anti-B7 mAbs and hCTLA4Ig therapy or genetic disruption of the CD28 molecule results in a diminished ability to generate a productive primary T cell response and severely suppresses T-dependent humoral responses. Under these, perhaps physiological, conditions, stimulation of primary T cell responses is largely dependent on B7-2-mediated costimulation. At early time points in the immune response, B7-2 is expressed constitutively on dendritic cells and functions to regulate both Th1 and Th2 responses. As the immune response progresses, both B7-1 and B7-2 are upregulated, resulting in increased

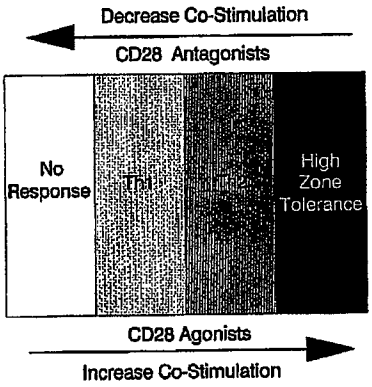

Figure 1 The strength-of-signal model.

costimulatory signals and an increased strength-of-signal that promotes T cell expansion and cytokine production and may skew the T cell response toward the Th2 phenotype. Thus, any reagent or situation that reduces costimulation during this response reduces the strength-of-signal, promoting Th1 responses. For example, the treatment of BALB/c mice with hCTLA4Ig during infection with *L. major* protected the normally susceptible strain from disease by decreasing the amount of IL-4 being produced (34). Blockade of CD28 signaling also inhibited the production of an IL-4 response to *H. polygyrus* and decreased humoral responses (35, 52, 98, 99). Finally, the effect of anti-B7-2 mAbs observed by Kuchroo et al may reflect the blockade of initial costimulation during the induction of R-EAE, thus skewing the response to the disease promoting Th1 phenotype (107).

Finally, high levels of costimulation coupled with high TCR occupancy may, in fact, downregulate immune responses. This effect may occur due to the extensive signaling via the TCR and CD28, as has been suggested as an explanation for "high zone tolerance" or "clonal exhaustion." Alternatively, hyperstimulation may substantially upregulate CTLA-4 on the activated T cells. Since the ligation of CTLA-4 inhibits immune responses, the interactions of CTLA-4 with either B7-1 or B7-2 may further amplify the suppression observed under these conditions. In this regard, in some tumor models the expression of both B7-1 and B7-2 on a tumor is less effective at inducing an immune response than is a tumor on which is expressed one or the other (T Gajewski, personal communication; CJ Bartels, & JC Yang, personal communication). Furthermore, transgenic mice expressing high levels of B7-1 on their B cells are profoundly deficient in their ability to receive T cell help for antibody production (111). Therefore, the careful manipulation of the CD28/B7 signaling pathway may dramatically impact the course of an immune response.

CONCLUSION

The studies summarized in this review demonstrate the importance of the CD28/B7 signaling pathway and also begin to illustrate its complexities. First, CD28 functions by costimulating T cells and preventing the induction of either anergy or apoptosis. Second, a CD28 homologue, CTLA-4, counterbalances CD28 by downregulating the immune response either by competing with CD28 for its ligands or by inducing apoptosis. Third, two distinct molecules B7-1 and B7-2 function as costimulatory ligands for CD28 and CTLA-4. While B7-2 dominates in primary responses, the roles of B7-1 and B7-2 during an ongoing response depend upon: the relative expression of CD28/CTLA-4; the nature and concentration of cytokines in the surrounding milieu; and the characteristics of the APC that are encountered. Finally, manipulation of the CD28/B7 pathway can alter the balance of the immune response by influencing the nature of the cytokines produced in response to antigen exposure. A better understanding of the consequences of regulation of the CD28/B7 costimulatory interactions will offer new insights into important elements controlling the immune response and allow for the development of novel immunotherapies for cancer, transplantation, and autoimmune disease.

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Binding of the B Cell Activation Antigen B7 to CD28 Costimulates T Cell Proliferation and Interleukin 2 mRNA Accumulation

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Summary

A successful immune response requires intercellular contact between T and B lymphocytes. We recently showed that CD28, a T cell surface protein that regulates an activation pathway, could mediate intercellular adhesion with activated B cells by interaction with the B7 antigen. Here we show that CD28 is the primary receptor for B7 on activated peripheral blood T cells, that CD28 binds to B7 in the absence of other accessory molecules, and that interaction between CD28 and B7 is costimulatory for T cell activation. To characterize the binding of CD28 to B7, we have produced genetic fusions of the extracellular portions of B7 and CD28, and immunoglobulin (Ig) Cγ1 chains. ^{125}I-labeled B7 Ig bound to CD28-transfected Chinese hamster ovary (CHO) cells, and to immobilized CD28 Ig with a K_d ~200 nM. B7 Ig also inhibited CD28-mediated cellular adhesion. The function of CD28-B7 interactions during T cell activation was investigated with soluble fusion proteins and with B7-transfected CHO cells. Immobilized B7 Ig and B7$^+$ CHO cells costimulated T cell proliferation. Stimulation of T cells with B7$^+$ CHO cells also specifically increased levels of interleukin 2 transcripts. These results demonstrate that the CD28 signaling pathway could be activated by B7, resulting in increased T cell cytokine production and T cell proliferation. Cellular interactions mediated by B7 and CD28 may represent an important component of the functional interactions between T and B lymphoid cells.

It has long been known that interactions between T and B lymphocytes play a central role in regulating an immune response (1, 2). More recent studies have shown that activation and differentiation of both T_H and B lymphocytes is dependent upon direct intercellular (cognate) interactions between these cell types. While the specificity of T_H cell–B cell interactions is determined by interaction between the TCR/CD3 complex (3–5) and antigen associated with class II MHC molecules on B cells (6), interactions between other (accessory) cell adhesion molecules are also necessary for a full immune response (7–9). Interactions between accessory receptors and their counter-receptors may increase the avidity of cellular interactions (7); control lymphocyte localization and migration (10); and have direct signaling functions (7, 11, 12) during lymphocyte activation. Accessory receptors and their counter-receptors involved in T_H–B cell interactions include (reviewed in reference 7): CD2 and LFA-3, CD4 and class II MHC molecules, LFA-1, and ICAM-1 and ICAM-2.

The T cell homodimer, CD28, a member of the Ig superfamily (13), has been shown in studies using mAbs to have an accessory function during T cell activation (14). Anti-CD28 mAbs have been shown to costimulate T cell proliferation induced by a number of polyclonal stimuli (reviewed in reference 14). These mAbs also inhibited alloantigen and soluble antigen-specific T cell responses (15, 16), indicating that CD28-mediated signalling may be crucial during these responses. CD28-mediated T cell activation, unlike that initiated via TCR, was resistant to inhibition by the immunosuppressive agent, cyclosporine (17). Some of the effects of anti-CD28 mAbs appear to result, in part, from the coordinate stimulation of several T cell–derived cytokines (18) through stabilization of their mRNAs (19).

We recently reported that Chinese hamster ovary (CHO)[1] cells transfected with CD28 mediated specific adhesion with certain activated normal and malignant B cells (20). CD28-mediated adhesion was blocked by the mAb BB-1 (21), which recognizes the B cell activation antigen, termed B7, another member of the Ig superfamily (22). COS cells transfected with a cDNA clone encoding B7 adhered specifically to CD28$^+$ CHO cells, thereby indicating that the B7 antigen is a counter-receptor for CD28.

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; dhfr, dihydrofolate reductase.

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The recognition of CD28 by B7 is a novel mechanism to regulate interaction of T and B lymphocytes. In this paper, we have further characterized biochemical and functional aspects of the interaction between these molecules. Fusion proteins of B7 and CD28 with human Ig Cγ1 chains were expressed and used to measure the specificity and apparent affinity of their interaction. We have also used purified B7Ig fusion protein, as well as CHO cells transfected with B7 to investigate the functional importance of this interaction on T cell activation and cytokine production.

Materials and Methods

Plasmid Construction. Expression plasmids containing cDNAs encoding CD28 (13), CD5 (23), and B7 (22) have been described previously. For initial attempts at expression of soluble forms of CD28 and B7, constructs were made (OMCD28 and OMB7) in which stop codons were introduced upstream of the transmembrane domains and the native signal peptides were replaced with the signal peptide from oncostatin M (24). These were made using synthetic oligonucleotides for reconstruction (OMCD28) or as primers for PCR (OMB7). CD28 Ig and B7 Ig fusion constructs were made in two parts. The 5' portions were made using OMCD28 and OMB7 as templates and the oligonucleotide, CTAGCCACTGAAGCTTCACCATGGGTGTACTGCTCACAC, (corresponding to the oncostatin M signal peptide) as a forward primer, and either TGGCATGGGCTCCTGATCAGGCTTAGAAGGTCCGGGAAA or, TTTGGGCTCCTGATCAGGAAAATGCTCTTGCTTGGTTGT as reverse primers, respectively. Products of the PCR reactions were cleaved with restriction endonucleases (HindIII and BclI) at sites introduced in the PCR primers and gel purified.

The 3' portion of the fusion constructs corresponding to human Ig Cγ1 sequences was made by a coupled reverse transcriptase (from Avian myeloblastosis virus; Life Sciences Inc., St. Petersburg, FL)-PCR reaction using RNA from a myeloma cell line producing human-mouse chimeric mAb L6 (P. Fell and M. Gayle, unpublished results) as template. The oligonucleotide, AAGCAAGAGCATTTTCCTGATCAGGAGCCCAAATCTTCTGACAAAACTCACACATCCCCACCGTCCCCAGCACCTGAACTCCTG, was used as forward primer, and CTTCGACCAGTCTAGAAGCATCCTCGTGCGACCGCGAGAGC as reverse primer. Reaction products were cleaved with BclI and XbaI and gel purified. Final constructs were assembled by ligating HindIII/BclI cleaved fragments containing CD28 or B7 sequences together with BclI/XbaI cleaved fragment containing Ig Cγ1 sequences into HindIII/XbaI cleaved CDM8. Ligation products were transformed into MC1061/p3 cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequencing (see Fig. 1). CD5 Ig was constructed in identical fashion, using CATTGCACAGTCAAGCTTCCATGCCCATGGGTTCTCTGGCCACCTTG, as forward primer and ATCCACAGTGCAGTGATCATTTGGATCCTGGCATGTGAC as reverse primer. The PCR product was restriction endonuclease digested and ligated with the Ig Cγ1 fragment as described above. The resulting construct (CD5 Ig) encodes a mature protein comprising residues 1–347 of CD5, two amino acids introduced by the construction procedure (amino acids DQ), followed by the Ig Cγ1 hinge region as shown in Fig. 1.

PCR reactions (0.1 ml final volume) were run in *Taq* polymerase buffer (Stratagene, Torrey Pines, CA), containing 20 μmol each dNTP; 50–100 pmol of the indicated primers; template (1 ng plasmid or cDNA synthesized from ≤1 μg total RNA using random hexamer primer); and *Taq* polymerase (Stratagene). Reactions were run on a thermocycler (Perkin Elmer Corp., Norwalk, CT) for 16–30 cycles (a typical cycle consisted of steps of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C).

Immunostaining and FACS Analysis. Transfected CHO cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (25) or BB-1 (21), or with Ig fusion proteins (all at 10 μg/ml in DMEM containing 10% FCS) for 1–2 h at 4°C. Cells were then washed, and incubated for an additional 0.5–2 h at 4°C with a FITC-conjugated second step reagent (goat anti–mouse Ig serum for murine mAbs, or goat anti–human Ig Cγ serum for fusion proteins; Tago, Inc., Burlingame, CA). Fluorescence was analyzed on a FACS IV® cell sorter (Becton Dickinson and Co., Mountain View, CA) equipped with a four decade logarithmic amplifier.

Cell Culture and Transfections. Stable transfectants expressing CD28, CD5, or B7 were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (20). Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μM. Lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by fluorescence-activated cell sorting from CD28-transfected populations.

COS cells were transfected with expression plasmids using a modification of the protocol of Seed and Aruffo (26). Cells were seeded at 10^6 per 10 cm diameter culture dish 18–24 h before transfection. Plasmid DNA was added (~15 μg/dish) in a volume of 5 mls of serum-free DMEM containing 0.1 mM cloroquine and 600 μg/ml DEAE Dextran, and cells were incubated for 3–3.5 h at 37°C. Transfected cells were then briefly treated (~2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16–24 h in DMEM containing 10% FCS. At ~24 h after transfection, culture medium was removed and replaced with serum-free DMEM (6 ml/dish). Incubation was continued for 3 d at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 d at 37°C, the spent medium was again collected and cells were discarded.

Purification of Ig Fusion Proteins. The first and second collections of spent serum-free culture media from transfected COS cells were used as source for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium was applied to a column (~200–400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, MA) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column was washed with 1 M potassium phosphate, pH 8, and bound protein was eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of A_{280} absorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28 Ig and B7 Ig, respectively, by amino acid analysis of solutions of known absorbance. The recovery of purified CD28 Ig and B7 Ig binding activities were nearly quantitative as judged by FACS® analysis after indirect fluorescent staining of B7⁺ and CD28⁺ CHO cells.

Cell Separation and Stimulation. PBL were isolated by centrifu-

gation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and cultured in 96-well, flat-bottomed plates (4×10^4 cells/well, in a volume of 0.2 ml) in RPMI containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by uptake of [^{3}H]thymidine during the last 5 h of a 3-d culture. PHA-activated T cells were prepared by culturing PBL with 1 μg/ml PHA (Wellcome, Charlotte, NC) for 5 d, and 1 d in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium before use.

Cell Surface Iodination and Immunoprecipitation. PHA-activated T cells were cell-surface labeled with ^{125}I using lactoperoxidase and H_2O_2 (27). Nonionic detergent extracts of labeled cells were prepared and subjected to immunoprecipitation analysis as described previously (28).

Radiolabeling of B7 Ig. Purified B7 Ig (25 μg) in a volume of 0.25 ml of 0.12 M sodium phosphate, pH 6.8 was iodinated using 2 mCi ^{125}I and 10 μg of chloramine T. After 5 min at 23°C, the reaction was stopped by the addition of 20 μg sodium metabisulfite, followed by 3 mg of KI and 1 mg of BSA. Iodinated protein was separated from unreacted ^{125}I by chromatography on a 5-ml column of Sephadex G-10 equilibrated with PBS containing 10% FCS. Peak fractions were collected and pooled. The specific activity of ^{125}I-B7 Ig labeled in this fashion was 1.5×10^6 cpm/pmol (2.8×10^7 cpm/μg).

B7 Ig was also metabolically labeled with [^{35}S]methionine. COS cells were transfected with a plasmid encoding B7 Ig as described above. At 24 h after transfection, [^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to concentrations of 115 μCi/ml) in DMEM containing 10% FCS and 10% normal levels of methionine. After incubation at 37°C for 3 d, medium was collected and used for purification of B7 Ig as described above. Concentrations of [^{35}S]methionine-labeled B7 Ig were estimated by comparison of staining intensity after SDS-PAGE with intensities of known amounts of unlabeled B7 Ig. The specific activity of [^{35}S]methionine-labeled B7 Ig was $\sim 2 \times 10^6$ cpm/μg.

Binding Assays. For assays using immobilized CD28Ig, 96-well plastic dishes were coated for 16–24 h with a solution containing CD28Ig (0.5 μg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were then blocked with binding buffer (DMEM containing 50 mM BES, pH 6.8, 0.1% BSA, and 10% FCS) (Sigma Chemical Co., St. Louis, MO) before addition of a solution (0.09 ml) containing ^{125}I-B7 Ig ($\sim 3 \times 10^5$ cpm) or [^{35}S]-B7 Ig (1.5×10^5 cpm) in the presence or absence of competitor. After incubation for 2–3 h at 23°C, wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantified by liquid scintillation or γ counting. When binding of ^{125}I-B7 to CD28$^+$ CHO cells was measured, cells were seeded (2.5×10^4/well) in 96-well plates 16–24 h before the start of the experiment. Binding was otherwise measured as described above.

SDS Page. SDS-PAGE was performed on linear acrylamide gradient gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X-ray film (Kodak XAR-5; Eastman Kodak Co., Rochester, NY).

RNA Blot Analysis. RNA was prepared from stimulated PHA blasts by a published procedure (29). Aliquots of RNA (20 μg) were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose by capillary action. RNA was crosslinked to the membrane by UV light in a Stratalinker (Stratagene), and the blot was prehybridized and hybridized with a ^{32}P-labeled probe for human IL-2 (prepared from a ~600-bp cDNA fragment provided by Dr. S. Gillis; Immunex Corp., Seattle, WA). Equal loading of RNA samples was verified both by rRNA staining and by hybridization with a rat glyceraldehyde-6-phosphate dehydrogenase probe (GAPDH, a ~1.2-kb cDNA fragment provided by Dr. A. Purchio, Oncogen).

Results

Construction and Expression of B7 and CD28 Immunoglobulin Cγ Fusion Proteins. In initial attempts to make soluble derivative of B7 and CD28, we made cDNA constructs encoding molecules truncated at the NH_2-terminal side of their transmembrane domains. In both cases, the native signal peptides were replaced with the signal peptide from oncostatin M (24), which mediates efficient release of secreted proteins in transient expression assays (J. Kallestad, P. S. Linsley, and W. Brady, unpublished observations). These cDNAs were cloned into an expression vector, transfected into COS cells, and spent culture medium was tested for secreted forms of B7 and CD28. In this fashion, we produced several soluble forms of B7, but in repeated attempts, we were unable to detect soluble CD28 molecules.

We then took the approach of making receptor Ig Cγ fusion proteins. Other investigators (23, 30) have shown these molecules to be efficiently produced in transient expression systems, and easily purified and detected using standard immunochemical techniques. The B7 and CD28 extracellular regions, preceded by the signal peptide to oncostatin M, were fused in frame to an Ig Cγ1 cDNA, as shown in Fig. 1 *A*. During construction, the Ig hinge disulfides were mutated to serine residues to abolish intrachain disulfide bonding. The resulting fusion proteins were produced in COS cells and purified by affinity chromatography on immobilized protein A as described in Materials and Methods. Yields of purified protein were typically 1.5–4.5 mg/liter of spent culture medium.

As shown in Fig. 1 *B*, the B7 Ig fusion protein migrated during SDS-PAGE under nonreducing conditions predominantly as a single species of M_r 70,000, with a small amount of material migrating as a M_r ~150,000 species. After reduction, a single M_r ~75,000 species was observed. The nature of the M_r ~150,000 species was not investigated further. CD28 Ig migrated as a M_r ~140,000 species under nonreducing conditions and a M_r ~70,000 species after reduction, indicating that it was expressed as a homodimer. Since the Ig Cγ1 hinge cysteines had been mutated, disulfide linkage probably involved cysteine residues which naturally form interchain bonds in the CD28 homodimer (25).

Binding Activities of B7 and CD28 Immunoglobulin Cγ Fusion Proteins. To investigate the functional activities of B7 Ig and CD28 Ig, we first tested binding to CHO cell lines expressing CD28 or B7. In early experiments, spent culture media from transfected COS cells was used as a source of fusion protein, while in later experiments, purified proteins were used (Fig. 2). Binding was detected by addition of FITC-conjugated goat anti-human Ig second step reagent. B7 Ig was bound by CD28$^+$ CHO, while CD28 Ig was bound by B7$^+$ CHO. B7 Ig also bound weakly to B7$^+$ CHO (Fig. 2), suggesting that this molecule has a tendency to form

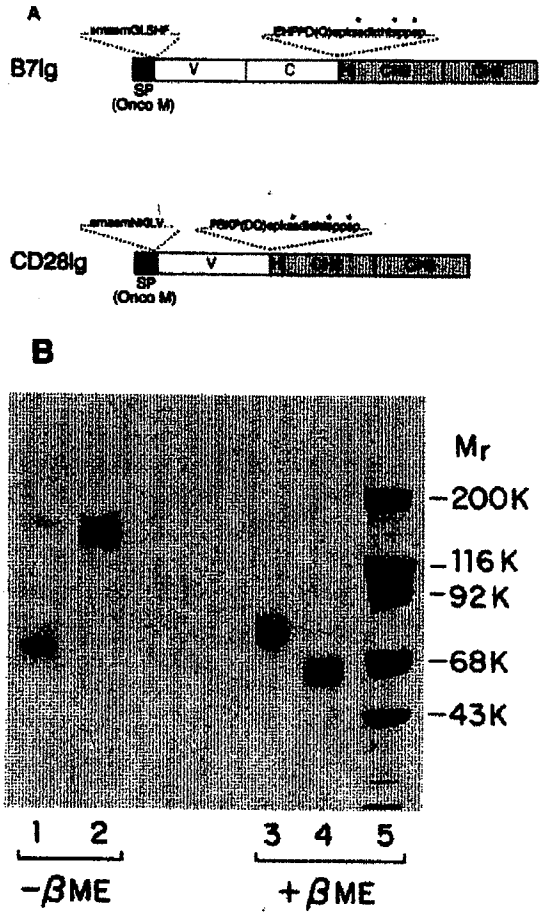

Figure 1. Construction and expression of B7 and CD28 Ig Cγ fusion proteins. (*A*) Maps of B7 Ig and CD28 Ig constructs. cDNA constructs encoding the indicated portions of oncostatin M (*dark shaded regions*), B7 and CD28 (*unshaded regions*), and human Ig Cγ1 (*stippled regions*) were constructed as described in Materials and Methods. Sequences displayed show the junctions between B7 and CD28 (*capital letters*), and the signal peptide (SP) of oncostatin M at their NH_2 termini, and the hinge (H) of Ig Cγ1 at their COOH termini. Amino acids introduced during construction are indicated in parentheses. Asterisks denote cysteine to serine mutations introduced in the hinge. Boxes labeled V and C denote Ig superfamily-like domains present in CD28 (13) and B7 (22). The CH2 and CH3 domains of Ig Cγ1 are also indicated. (*B*) Purification of B7 Ig and CD28 Ig. Expression plasmid constructs encoding the fusion proteins displayed above were transfected into COS cells. Serum-free conditioned medium was collected, and Ig Cγ-containing proteins were purified by protein A affinity chromatography as described in Materials and Methods. Aliquots (1 μg) of B7 Ig (lanes *1* and *3*) or CD28 Ig (lanes *2* and *4*) were subjected to SDS-PAGE (4–12% acrylamide gradient) under nonreducing (− βME, lanes *1* and *2*) or reducing (+ βME lanes *3* and *4*) conditions. Lane *5* shows mol wt markers. Proteins were visualized by staining with Coomassie Brilliant Blue.

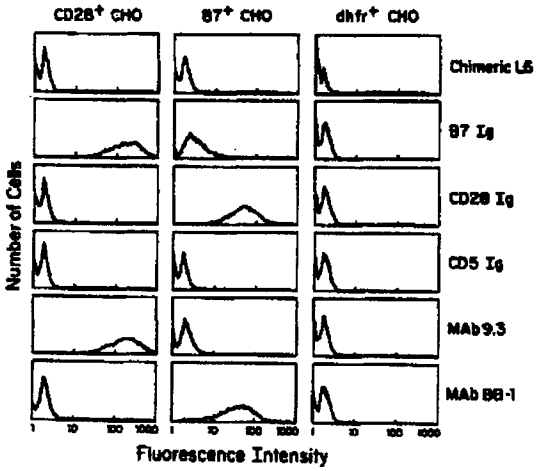

Figure 2. B7 Ig and CD28 Ig bind to transfected CHO Cells. Amplified transfected CHO cells expressing CD28, B7, or dhfr only (no surface marker) were first stained with human Ig Cγ1-containing proteins (chimeric mAb L6, CD28 Ig, B7 Ig, or CD5 Ig), or mouse mAbs (9.3 or BB-1 at 10 μg/ml), followed by FITC-conjugated anti-human or mouse Ig second step reagents. A total of 10,000 stained cells was then analyzed by FACS®.

homophilic interactions. No binding was detected of a chimeric mAb containing human Ig Cγ1, or of another fusion protein, CD5 Ig. Thus, B7 Ig and CD28 Ig retain binding activity for their respective counter-receptors.

We next determined the apparent affinity of interaction between B7 and CD28. B7 Ig was either metabolically labeled with [^{35}S]methionine, or iodinated, and radiolabeled derivatives were tested for binding to immobilized CD28 Ig or to CD28$^+$ CHO cells. A competition binding experiment using ^{125}I-B7 Ig and immobilized CD28 Ig is shown in Fig. 3 *A*. Binding of ^{125}I-B7 Ig was competed in dose-dependent fashion by unlabeled B7 Ig, and by mAbs 9.3 and BB-1. mAb 9.3 was the most effective competitor (half-maximal inhibition at 4.3 nM), followed by mAb BB-1 (half-maximal inhibition at 140 nM) and B7 Ig (half-maximal inhibition at 280 nM). Thus, mAb 9.3 was ~65-fold more effective as a competitor than B7 Ig, indicating that the mAb has greater apparent affinity for CD28. The same relative difference in avidities was seen when [^{35}S]methionine-labeled B7 Ig was used. Chimeric mAb L6 did not significantly inhibit binding; the inhibition at high concentrations in Fig. 3 *A* was not seen in other experiments. When the competition data were plotted in the Scatchard representation (Fig. 3 *B*), a single class of binding sites was observed (K_d ~200 nM). An identical K_d was detected for binding of ^{125}I-B7 Ig to CD28$^+$ CHO cells. Thus, both membrane bound CD28 and immobilized CD28 Ig showed similar apparent affinities for ^{125}I-B7.

CD28 is the Primary B7-binding Protein on PHA-activated T Cells. Although B7 Ig bound to immobilized CD28 Ig, and to CD28$^+$ CHO cells, it was not known whether B7 Ig could bind to CD28 naturally expressed on T cells. This is

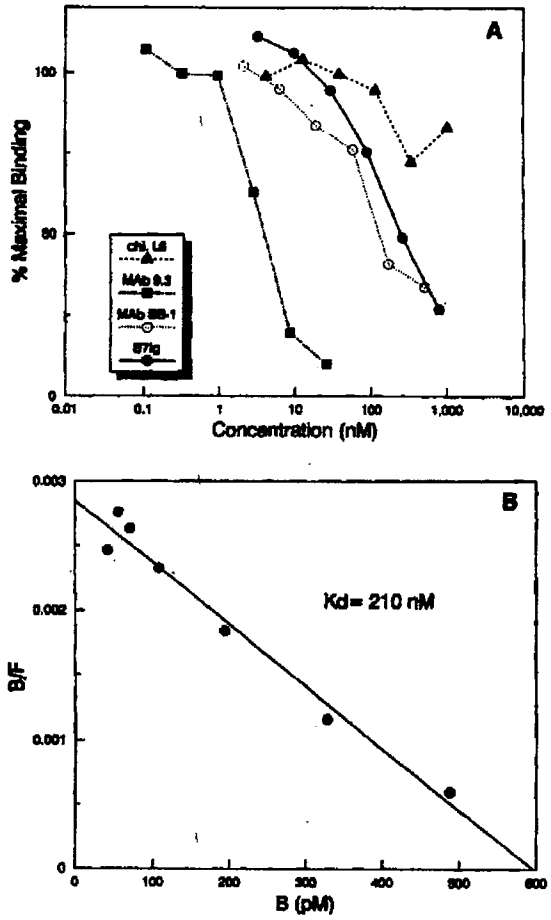

Figure 3. ^{125}I-labeled B7 Ig binds with high affinity to immobilized CD28 Ig. (*A*) Competition binding analysis of ^{125}I-B7 Ig. 96-well plastic dishes were coated with CD28 Ig as described in Materials and Methods. ^{125}I-labeled B7 Ig (3.3×10^6 cpm, 2×10^6 cpm/pmol) was then added to a concentration of 24 nM in the presence of the indicated concentrations of unlabeled chimeric L6, mAb 9.3, mAb BB-1, or B7 Ig. Plate-bound radioactivity was determined and is expressed as a percentage of radioactivity bound to wells treated without competitor (7,800 cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by ≤20%. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7 Ig. (*B*) Scatchard analysis of unlabeled B7 Ig competition binding experiment. Data shown in Fig. 3 *A* was replotted in the Scatchard representation and a binding constant (K_d) was estimated from the slope of the line best fitting the experimental data ($r = -0.985$).

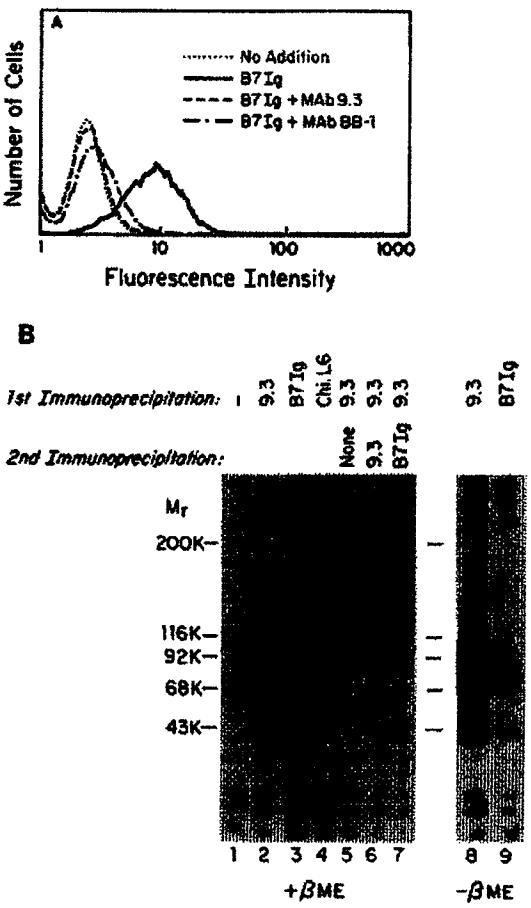

Figure 4. CD28 is the primary B7 Ig binding protein on PHA-activated T cells. (*A*) FACS® profiles of B7 Ig binding to PHA blasts. PHA-stimulated PBL were stained without or with B7 Ig at 10 μg/ml. Where indicated mAbs 9.3 or BB-1 were added (also at 10 μg/ml) to cells simultaneously with B7 Ig. Bound mAb was detected with a FITC-conjugated goat anti-human Ig Cγ reagent, and stained cells were analyzed by FACS®. (*B*) Autoradiogram of ^{125}I-labeled proteins immunoprecipitated by B7 Ig. PHA-stimulated PBL were surface labeled with ^{125}I as described in Materials and Methods. Aliquots of a nonionic detergent extract of labeled cells ($\sim 3 \times 10^6$ cpm in a volume of 0.12 ml) were subjected to immunoprecipitation analysis with no addition (lane *1*), mAb 9.3 (5 μg, lane *2*), B7 Ig (10 μg, lane *3*), or chimeric L6 (10 μg, lane *4*). After precipitation of antigen-antibody complexes using formalin-fixed *Staphylococcus aureus*, extracts were then subjected to a second immunoprecipitation analysis with no addition (lane *5*), mAb 9.3 (5 μg, lane *6*), or B7 Ig (10 μg, lane *7*). Washed immunoprecipitates were analyzed by SDS-PAGE (5–15% acrylamide gradient) under reducing (+ β-ME, lanes *1–7*) or non-reducing conditions (− β-ME, lanes *8* and *9*), the gel was dried, and subjected to autoradiography.

an important consideration since the level of CD28 on transfected cells was ~10-fold higher than that found on PHA-activated T cells (20). We therefore tested PHA-activated T cells for binding of B7 Ig by FACS® analysis after indirect immunofluorescent staining. As shown in Fig. 4 *A*, these cells bound significant levels of B7 Ig, and binding was inhibited by mAbs 9.3 and BB-1 We also determined the identity of B7 Ig–binding proteins by immunoprecipitation analysis of ^{125}I-surface-labeled cells (Fig. 4 *B*). Both mAb 9.3 and B7 Ig immunoprecipitated a M_r ~45,000 protein under reducing conditions and M_r ~45,000 and ~90,000 proteins

under nonreducing conditions, with the latter form being more prominent. The M_r ~45,000 protein found in the sample precipitated with chimeric mAb L6 was due to spillover and was not seen in other experiments. mAb 9.3 was more effective at immunoprecipitation than B7 Ig, in agreement with the greater affinity of the mAb (Fig. 3). Identical results were obtained when CD28+ CHO cells were used for immunoprecipitation analysis. Preclearing of CD28 by immunoprecipitation with mAb 9.3 also removed B7 Ig-precipitable material, indicating that both mAb 9.3 and B7 Ig bound the same ^{125}I-labeled protein. Taken together, the results in this section indicate that CD28 is the major receptor for B7 Ig on PHA-activated T cells.

B7-Binding to CD28 Blocks CD28-mediated Adhesion. mAbs to CD28 have potent biological activities on T cells, suggesting that interaction of CD28 with its natural ligand(s) may also have important functional consequences. As a first step in determining functional consequences of interaction between B7 and CD28, we asked whether B7 Ig could block the CD28-mediated adhesion assay (20). As shown in Fig. 5, B7 Ig blocked CD28-mediated adhesion somewhat less effectively than mAb 9.3 (half-maximal inhibition at 200 nM as compared with 10 nM for mAb 9.3). The relative effectiveness of these molecules at inhibiting CD28-mediated adhesion was similar to their relative binding affinities in competition binding experiments (Fig. 3 *A*). CD28 Ig failed to inhibit CD28-mediated adhesion at concentrations of up to 950 nM (data not shown), suggesting that much higher levels of CD28 Ig were required to compete with the high local concentrations of CD28 present on transfected cells.

B7 Costimulates T Cell Proliferation. We next investigated whether triggering of CD28 by B7 was costimulatory for T cell proliferation. The ability of B7 Ig to costimulate proliferation of PBL together with anti-CD3 was first explored. In several experiments, B7 Ig in solution at concentrations of 1–10 μg/ml showed only a modest enhancement of prolifer-

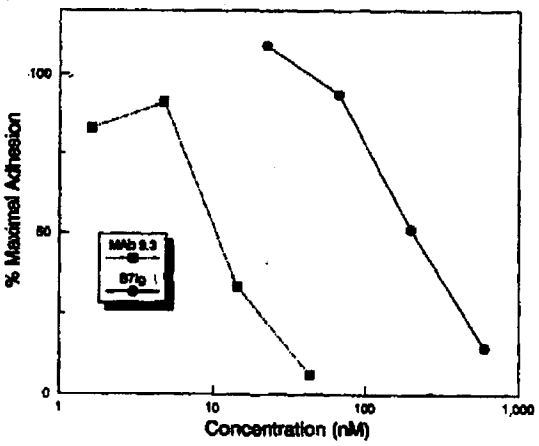

Figure 5. B7 Ig specifically inhibits CD28-mediated adhesion. The adhesion of ^{51}Cr-labeled PM lymphoblastoid cells to monolayers of CD28+ CHO cells was measured as described previously (20) in the presence of the indicated amounts of mAb 9.3 or B7 Ig. Data are expressed as a percentage of cells bound in the absence of competitor (40,000 cpm or ~1.1 × 10^5 cells). Each point represents the mean of triplicate determinations; coefficients of variation were <25%.

Table 1. *B7 Ig Is Costimulatory with Anti-CD3 for Proliferation of Peripheral Blood Lymphocytes*

Exp.	CD28 stimulation	[^{3}H]-T incorporation	
		− Anti-CD3	+ Anti-CD3
		cpm × 10^{-3}	
1	None	0.1	26.0
	mAb 9.3 (solution)	0.3	156.1
	mAb 9.3 (immobilized)	0.1	137.4
	B7Ig (immobilized)	0.1	174.5
2	None	0.2	19.3
	mAb 9.3 (solution)	0.4	75.8
	B7+ CHO cells	9.4	113.9
	dhfr+CHO cells	23.8	22.1

PBL were isolated and cultured in the presence of the indicated costimulators of T cell proliferation. Anti-CD3 stimulation was with mAb G19-4 at 1 μg/ml in solution. For CD28 stimulation, mAb 9.3 or B7 Ig were added in solution at 1 μg/ml, or after immobilization on the culture wells by pre-incubation of proteins at 10 μg/ml in PBS for 3 h at 23°C and then washing the culture wells. B7+ CHO and control dhfr+ CHO cells were irradiated with 1,000 rad before mixing with PBL at a 4:1 ratio of PBL/CHO cells. After culture for 3 d, proliferation was measured by uptake of [^{3}H]thymidine for 5 h. Values shown are means of determinations from quadruplicate cultures (SEM <15%).

Table 2. *B7 is Directly Stimulatory for Proliferation of PHA Blasts*

T cells/CHO cells	[^{3}H]-T incorporation	
	+B7+ CHO	+CD5+ CHO
	cpm × 10^{-3}	
25:1	92.7	15.5
50:1	135.4	19.5
100:1	104.8	16.8
200:1	90.3	17.7
400:1	57.0	13.7
800:1	42.3	17.6

PHA blasts were cultured at 50,000 cells/well with varying amounts of irradiated CHO cell transfectants. After 2 d of culture, proliferation was measured by a 5-h pulse of [^{3}H]thymidine. Shown are means of quadruplicate determinations (SEM <15%). Background proliferation of PHA blasts without added CHO cells was 11,200 cpm. [^{3}H]Thymidine incorporation by irradiated B7+ CHO and CD5+ CHO cells alone was <1,800 cpm at each cell concentration and has been subtracted from the values shown.

Table 3. *Stimulation of Proliferation of PHA Blasts by B7⁺ CHO Cells Is Inhibited by CD28- or B7- specific mAbs*

Stimulation	mAb	[³H]-T incorporation
		cpm × 10⁻³
None	None	10.8
B7⁺ CHO	None	180
B7⁺ CHO	9.3 Fab	132
B7⁺ CHO	BB-1	98.3
B7⁺ CHO	LB-1	196
CD7⁺ CHO	None	11.5
CD7⁺ CHO	9.3 Fab	10.0
CD7⁺ CHO	BB-1	10.0
CD7⁺ CHO	LB-1	11.3

PHA blasts were stimulated as described in Table 2 with irradiated CHO cells at a ratio of 100:1 T cells/CHO cells. mAbs were added at 10 μg/ml at the beginning of culture. mAb LB-1 (21) is an isotype-matched control for mAb BB-1. Proliferation was measured by uptake of [³H]-thymidine during a 5-h pulse after 2 d of culture. Values represent means of quadruplicate cultures (SEM <15%).

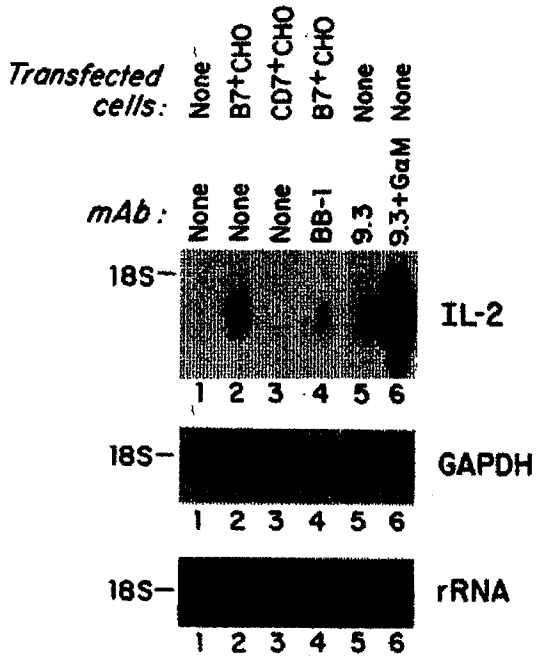

Figure 6. B7 stimulates accumulation of IL-2 mRNA. PHA blasts (5×10^7) were mixed with transfected CHO cells (at a ratio of 40:1 T cells/CHO cells), and/or mAbs as indicated. mAb 9.3 was used at 10 μg/ml. mAb BB-1 (20 μg/ml) was added 1 h before addition of B7⁺ CHO cells. When mAb 9.3 was crosslinked, goat anti-mouse Ig (40 μg/ml) was added 10 min after addition of mAb 9.3. Cells were incubated for 6 h at 37°C, RNA was isolated and subjected to RNA blot analysis, using ^{32}P-labeled IL-2 or GAPDH probes.

ation even though the anti-CD28 mAb 9.3 was effective. Because CD28 crosslinking has been identified as an important determinant of CD28 signal transduction (31), B7 Ig was also compared to 9.3 when immobilized on plastic wells (Table 1, Exp. 1). Under these conditions, B7 Ig was able to enhance proliferation and compared favorably with mAb 9.3. B7⁺ CHO cells also were tested and compared with control dhfr⁺ CHO cells for costimulatory activity on resting lymphocytes (Table 1, Exp. 2). In this experiment, proliferation was seen with dhfr⁺ CHO cells in the absence of anti-CD3 mAb because of residual incorporation of [³H]thymidine after irradiation of these cells (data not shown). This stimulation was not enhanced by anti-CD3 mAb and was not seen in other experiments (Tables 2 and 3) where transfected CHO cells were added at lower ratios. However, B7⁺ CHO cells were very effective at costimulation with anti-CD3 mAb, indicating that cell surface B7 had similar activity in this assay as anti-CD28 mAbs.

We also tested whether B7⁺ CHO cells could directly stimulate proliferation of resting PHA blasts which respond directly to CD28 crosslinking by mAb 9.3. Again, the B7⁺ CHO cells were very potent in stimulating proliferation (Table 2) and were able to do so at very low cell numbers (PHA blast:B7⁺ CHO ratios of >800:1). The control CD5⁺ CHO cells did not possess a similar activity. (In a number of different experiments neither dhfr⁺ CHO, CD5⁺ CHO, nor CD7⁺ CHO cells stimulated T cell proliferation. These were therefore used interchangeably as negative controls for effects induced by B7⁺ CHO cells). The stimulatory activity of B7⁺ CHO was further shown to result from CD28/B7 interaction, since mAb BB1 inhibited stimulation by the B7⁺ CHO cells without affecting background proliferation in the presence of CD7⁺ CHO cells (Table 3). mAb LB-1 (21), an IgM mAb to a different B cell antigen, did not inhibit proliferation. mAb 9.3 (Fab fragments) inhibited proliferation induced by B7⁺ CHO but not background proliferation seen with CD7⁺ CHO cells. The experiments show that B7 is able to stimulate signal transduction and augment T cell activity by binding to CD28, but that crosslinking is required and B7 expressed on the cell surface is most effective.

B7 Stimulates IL-2 mRNA Accumulation. We also investigated effects of CD28/B7 interactions on IL-2 production by analyzing transcript levels in PHA-blasts stimulated with B7⁺ CHO cells or CD7⁺ CHO cells. RNA was prepared from stimulated cells and tested by RNA blot analysis for the presence of IL-2 transcripts (Fig. 6). B7⁺ CHO cells, but not CD7⁺ CHO cells, induced accumulation of IL-2 transcripts. Induction by B7⁺ CHO cells was partially blocked by mAb BB-1. Induction by B7⁺ CHO cells was slightly better than achieved by mAb 9.3 in solution, but less effective than mAb 9.3 after crosslinking with goat anti-mouse Ig. Thus, triggering of CD28 by cell surface B7 on apposing cells stimulated IL-2 mRNA accumulation.

Discussion

We have used soluble Ig Cγ fusions of both CD28 and B7 to measure the strength of their interaction. The apparent

K_d value for this interaction (~200 nM) is within the range of affinities observed for mAbs (2–10,000 nM; reference 32) and compares favorably with the affinities estimated for other lymphoid adhesion molecules. Schneck et al. (33) estimated the affinity (K_d ~100 nM) between a murine T cell hybridoma TCR and soluble alloantigen (class I MHC molecules). A K_d of 400 nM was measured between CD2 and LFA3 (34). The affinity of CD4 for class II MHC, while not measured directly, was estimated (35) to be ≥10,000 times lower than the affinity of gp120-CD4 interactions (K_d = 4 nM; reference 36). Thus, the affinity of B7 for CD28 appears greater than affinities reported for some other lymphoid adhesion systems.

The degree to which the apparent K_d of CD28/B7 interaction reflects their true affinity, as opposed to their avidity, depends on the valency and/or aggregation of the fusion protein preparations. In preliminary experiments, we examined the degree of aggregation of these preparations by size fractionation (TSK G3000SW column eluted with PBS). Under these conditions, B7 Ig eluted at M_r ~350,000, and CD28 Ig at M_r ~300,000. Both proteins thus behaved in solution as larger molecules than they appeared by SDS-PAGE (Fig. 1), suggesting that they may form higher aggregates. Alternatively, these results may indicate that both fusion proteins assume extended conformations in solution, resulting in large Stokes radii. Regardless, the interaction we measured using soluble proteins probably underestimates the true avidity between CD28 and B7 in their native membrane-associated state.

The relative contribution of different adhesion systems to the overall strength of T cell–B cell interactions is not easily gauged, but is likely a function of both affinity/avidity and the densities on apposing cell surfaces of the different receptors and counter-receptors involved. Since both CD28 and B7 are found at relatively low levels on resting lymphoid cells (16, 22), they may be less involved than other adhesion systems (7) in initiating intercellular interactions. The primary role of CD28/B7 interactions may be to maintain or amplify a response subsequent to induction of these counter-receptors on their respective cell types.

While occupancy or crosslinking of the T cell antigen receptor is sufficient to initiate a T cell immune response, full activation requires additional costimulatory signals. In some cases, soluble molecules such as IL-1 can provide costimulatory activity, but in other cases the nature of the molecule(s) involved is unclear (8, 9). Schwartz (9) has recently reviewed evidence for a costimulatory activity on antigen-presenting cells which may determine whether TCR occupancy leads to a productive or anergic response in murine T_H clones. This activity appears to require cell-cell contact, leads to increased IL-2 production, and may operate through a different signal-transducing pathway than the TCR (9). All of these characteristics are shared with the CD28 pathway (reviewed in reference 14), leading to the intriguing possibility that CD28 may be one of these functionally defined costimulatory molecules. In agreement with this possibility, is the finding by Ledbetter et al. (31) that anti-CD28 mAb can partially overcome inhibition of T cell proliferation induced by internalization of the TCR. Studies of the involvement of CD28/B7 interactions in various experimental systems will be required to fully evaluate the role of these costimulatory interactions.

Binding of B7 to CD28 on T cells was costimulatory for T cell proliferation (Tables 1–3), suggesting that some of the biological effects of anti-CD28 mAbs result from their ability to mimic T cell activation resulting from natural interaction between CD28 and its counter-receptor, B7. mAb 9.3 has greater affinity for CD28 than does B7 Ig (Figs. 4 and 5), which may account for the extremely potent biological effects of this mAb (14) in costimulating polyclonal T cell responses. Surprisingly, however, anti-CD28 mAbs are inhibitory for antigen-specific T cell responses (15, 16). This may indicate that antigen-specific T cell responses are dependent upon costimulation via CD28/B7 interactions, and that inhibition therefore results from blocking of CD28 stimulation. Despite the inhibition, CD28 must be bound by mAb under these conditions, implying that triggering by mAb is not always equivalent to triggering by B7. Although mAb 9.3 has higher apparent affinity for CD28 than B7 (Fig. 3), it may be unable under these circumstances to induce the optimal degree of CD28 clustering (31) for stimulation.

CD28/B7 interactions may also be important for B cell activation and/or differentiation. We have recently observed that mAbs 9.3 and BB-1 block T_H cell–induced Ig production by B cells (N. Damle, P. S. Linsley, and J. A. Ledbetter, unpublished results). This blocking effect may be due in part to inhibition by these mAbs of production of T_H-derived B cell–directed cytokines, but may also involve inhibition of B cell activation by interfering with direct signal transduction via B7. These results suggest that cognate activation of B lymphocytes, as well as T_H lymphocytes, is dependent upon interaction between CD28 and B7.

The mechanism of IL-2 transcript induction in T cells by $B7^+$ CHO cells (Fig. 6) is not currently known. It is well known that mRNAs for cytokines and other inflammatory mediators are characterized by the presence in their 3′ untranslated regions of AU-rich motifs (37), which control mRNA stability (38). Studies by Lindsten et al. (19) showed that anti-CD28 mAbs stimulated production of several T cell–derived cytokines by stabilizing transcripts for these molecules. However, as discussed by June et al. (14), anti-CD28 mAbs may also enhance transcription of IL-2 mRNA. Whatever the mechanism of transcript accumulation by CD28/B7 interactions, regulation of cytokine production by cell-cell contact has also been shown in other systems (39). Many effector functions of T lymphocytes are cytokine mediated (40), and control points in the synthetic pathways of these molecules are therefore extremely important. As dysfunctional cytokine production has been implicated as a contributing factor in several human diseases (40), it will be important to determine how B7/CD28 interactions regulate T cell cytokine production in various pathological states.

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Costimulatory Requirements of Naive CD4$^+$ T Cells

ICAM-1 or B7–1 Can Costimulate Naive CD4 T Cell Activation but Both Are Required for Optimum Response[1]

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Efficient initiation of a CD4 T cell response requires both activation through the TCR and costimulation provided by molecules on APC with counterreceptors on the T cell. We investigated the relative contribution of the ICAM-1:LFA-1 and B7:CD28/CTLA-4 costimulatory pathways in naive T cell activation, using either anti-CD28 Ab or fibroblast cell lines transfected with I-E^k, which express either no costimulatory molecules, ICAM-1 alone, B7–1 alone, or ICAM-1 and B7–1 together. Peptide Ag or immobilized anti-CD3 was used to provide the TCR signal. CD4 T cells from mice transgenic for the Vβ3/Vα11 TCR, which recognize a peptide of pigeon cytochrome *c* complexed to I-E^k, were used as a source of naive T cells. Naive T cells stimulated with Ag or anti-CD3 responded well to high numbers of APC expressing either ICAM-1 alone or B7–1 alone. However, APC expressing both ICAM-1 and B7–1 were much better stimulators of proliferation and IL-2 secretion at low cell numbers, and were far superior inducers of IL-2 at higher numbers, indicating a synergy between the two pathways. Stimulation provided by ICAM-1 could not be solely attributed to adhesive strengthening of other pathways, since costimulation was seen when immobilized anti-CD3 was used and when ICAM-1 only APC were added, indicating that ICAM-1 was in fact acting as a classic costimulatory molecule. Both the magnitude of the response and the amount of costimulation required for response were dependent on the intensity of TCR interaction. These results suggest that an efficient naive T cell response requires both a strong TCR signal and more than one costimulatory signal that will synergize with the TCR signal. This offers an explanation as to why APC such as dendritic cells and activated B cells, which express high levels of multiple costimulatory/adhesion molecules, are the only APC that elicit naive T cell responses. *The Journal of Immunology,* 1995, 155: 45–57.

The extent of the primary response of naive CD4 T cells, which involves their activation, expansion, and differentiation, will determine whether a successful immune response is mounted to a pathogen. It has been suggested that T cells require two signals for response: the first signal, which confers specificity on the response, is normally delivered via the TCR by its recognition of an antigenic peptide presented in the context of the MHC. The second signal, termed the "costimulus," is provided by one or more distinct cell surface molecules expressed by APC interacting with coreceptors on the T cells (1–3). Largely based on studies using Abs to the TCR, CD3 or CD2, it is thought that naive cells have very stringent requirements for activation and are rendered unresponsive or anergic if TCR ligation occurs in the absence of such "costimulatory" signals (1–6). These signals are neither Ag specific, nor MHC restricted, yet they may be critical for the induction of maximal T cell proliferation, cytokine production, and development of effector function. A surprisingly large number of APC surface molecules have been suggested to be involved in transmitting activation signals to counter receptors on CD4 T cells, including B7 (both B7–1 and B7–2) interacting with CD28/CTLA-4, ICAM-1 with LFA-1, LFA-3 (CD48 in the mouse) with CD2, HSA with an unknown receptor, and VCAM-1 with VLA-4 (7–17). The expression of some of these molecules is known to vary among different APC types and can also be up-regulated or induced by activation or by cytokines (11, 18–23).

Because of the low numbers of naive T cells specific for any particular protein Ag in unprimed animals, most of the conclusions about the nature of costimulatory molecules have been based on the analysis of polyclonal T cell activation by anti-CD3 or anti-TCR Abs or lectins, alloantigen-induced responses, and on studies with cloned cell

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lines. Moreover, most reports utilized resting T cells from unprimed mice or resting peripheral blood human T cells, which consist of a mixture of naive and memory cells. Therefore, the requirements for Ag-specific stimulation of naive CD4 T cells are unclear.

To directly assess the response of naive cells, we used a transgenic mouse model that provides a homogeneous source of naive CD4 T cells with a predetermined specificity for a peptide Ag of cytochrome *c* (PCCF) not present during their development, thereby ensuring that the cells are truly naive (24). We have previously demonstrated the naive status of the Tg CD4 T cells and have found fundamental differences between APC in their abilities to stimulate these CD4 cells (24, 25). Naive cells were induced to proliferate and secrete IL-2 only by dendritic cells and activated B cells, APC known to express high levels of several costimulatory molecules, including B7–1, B7–2, and ICAM-1 (11, 18–23). APC that express low levels or no costimulatory molecules, such as resting B cells and resting macrophage failed to activate naive T cells (24). As activated B cells and dendritic cells express a spectrum of different costimulatory molecules at the cell surface, it would be difficult to ascertain the contributions of the individual molecules in naive T cell activation using such APC. Therefore, we have developed a panel of transfected fibroblasts with more limited expression of potential costimulatory molecules to analyze the significance of individual pathways.

In this report, we investigate in detail the role of B7–1 and ICAM-1 in naive T cell activation. Costimulation was provided using either anti-CD28 Ab or three fibroblast cell lines transfected with I-E^k (and thus able to present PCCF) that express either B7–1 alone, ICAM-1 alone, or B7–1 and ICAM-1 together. We also tested sorted fibroblasts that expressed neither B7–1 nor ICAM-1. Our results confirm that naive CD4 T cell activation (proliferation and IL-2 secretion) is critically dependent on costimulatory signals in addition to signals through the TCR, with naive cells not responding to TCR stimulation alone. Using either the peptide Ag or polyclonal stimulation (anti-CD3) as a TCR signal, we show that effective costimulation can be provided individually by B7–1 or ICAM-1 but that they synergize to give maximum T cell activation. Furthermore, we find that activation represents an integration of TCR and costimulatory molecule signaling, with less costimulation required when the TCR signal is maximized.

Materials and Methods

Mice

H-$2^{b/k}$ Vβ3/Vα11 TCR transgenic mice were bred in the animal facilities at the University of California, San Diego and used at 2 to 4 mo of age. Mice were originally on a C57BL/6 × SJL background (26), but were backcrossed greater than four times to C57BL/6. Transgenic (H-$2^{b/b}$) males were then bred to B10.Br females to produce transgenic H-$2^{b/k}$ offspring.

T cells

Purified splenic $CD4^+$ T cells were isolated essentially as previously described (27). Briefly, spleen cells from transgenic H-$2^{b/k}$ mice were passed over nylon columns, treated with anti-CD8 (H02.2 and 3.155), anti-HSA (J11D), and anti-MHC class II (M.5114 and CA4.2.12), followed by a mouse anti-rat κ chain (MAR 18.5) and complement. High-density resting $CD4^+$ T cells (80% fraction) were isolated using discontinuous Percoll gradient centrifugation (4 layers, 45, 53, 62, and 80%). Residual APC were removed by plastic adherence. Remaining cells were >95% $CD4^+$ cells, 90 to 95% of which had a naive phenotype ($CD45RB^{high}$, Mel-14^{high}, Pgp-1^{low}) and expressed the transgenic TCR identified by staining with Ab to Vβ3 as previously shown (24). B cell contamination was less than 2%. For the anti-CD3 experiments, the CD4 cells were then isolated by magnetic separation based on CD45RB expression by sequential labeling with rat anti-mouse CD45RB (purified from the 23G2 hybridoma line, a kind gift from Dr. B. Puré, Rockefeller University, New York, NY), biotinylated mouse anti-rat κ (RG7.9.1), FITC-streptavidin (Zymed, San Francisco, CA), and biotinylated magnetic beads (Miltenyi Biotech, Sunnyvale, CA). Naive cells, positively selected on magnetic columns (MACS, Miltenyi Biotech) were >95% $CD45RB^{high}$ as previously shown (24).

Monoclonal antibodies

Phenotypic analysis. Cell staining and analysis were done on the four fibroblast cell lines. A panel of mAb recognizing murine cell surface markers was added at approximatively 10 μg/ml to aliquots of 5×10^5 cells. These included hamster IgG anti-ICAM-1 (3E2B) (a kind gift from Dr. A. Brian, La Jolla Cancer Research, La Jolla, CA), CTLA4-Ig (a kind gift from Dr. P. Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) (28), rat IgG2a biotinylated anti-B7–1 (1G10) (PharMingen, San Diego, CA), and rat IgG2b anti-class II (M5.114, from American Type Culture Collection (ATCC), Rockville, MD). As control mAb, hamster IgG (PharMingen), human IgG1 (Chemicon, Temecula, CA), rat IgG2a biotinylated (PharMingen), and rat IgG2b (PharMingen) were used respectively. Appropriate fluoresceinated secondary reagents were added after primary reagents were washed off in PBS/5% FCS/.01% NaN3. They included goat anti-hamster IgG (Caltag, San Francisco, CA), goat anti-human IgG (Caltag), PE-streptavidin (PharMingen), and RG7.9.1 (mouse anti-rat κ chain, ATCC). Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using Lysis II software.

For blocking studies, anti-LFA-1 mAb FD441.8 (rat IgG2b, ATCC) was purified from hybridoma culture supernatant by protein A affinity chromatography. A rat IgG2b mAb (PharMingen) was used as a control. CTLA4-Ig and a control human mouse chimeric mAb L6 were kindly provided by Dr. P. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute) (28). Anti-CD28 ascites was prepared with 37.51 cells (kindly provided by Dr. J. Allison, University of California, Berkeley, CA) in pristane-primed nude mice.

Fibroblast cell lines. Four related murine fibroblast cell lines were used as APC. The original fibroblast cell line was transfected with I-E^k (DCEK.Hi7, originally made by Dr. J. Miller and Dr. R. Germain, National Institutes of Health, Bethesda, MD, unpublished observations). The DCEK.Hi7 line was then additionally transfected by Dr. P. Kuhlman (La Jolla Cancer Research) (29) with ICAM-1 to yield the DCEK.ICAM line. Both of these cell lines constitutively express B7–1 (see Fig. 1). The third fibroblast cell line (DCEK.$B7^-$) was derived from the DCEK.ICAM line by FACS sorting of cells lacking B7 expression by indirect immunofluorescence. Lines of B7 negative APC were cultured for a few weeks and further cloned by limiting dilution. Several cell lines expressing MHC class II molecules and ICAM-1 and negative for B7 were selected for use (Fig. 1). One of these cell lines, designated DCEK.$B7^-$, was used in these studies. Finally, the control cell line (DCEK) was obtained from the DCEK.Hi7 line by FACS sorting of cells lacking B7 expression. These cells expressing MHC class II molecules were negative for B7 and ICAM-1 (Fig. 1).

For presentation, the APC lines were prepulsed with 20 μM peptide Ag, pigeon cytochrome *c* fragment (PCCF)[3] 88–104, purified by HPLC from whole pigeon cytochrome *c* (Sigma Chemical Co., St. Louis, MO)

[3] Abbreviation used in this paper: PCCF, pigeon cytochrome *c* fragment.

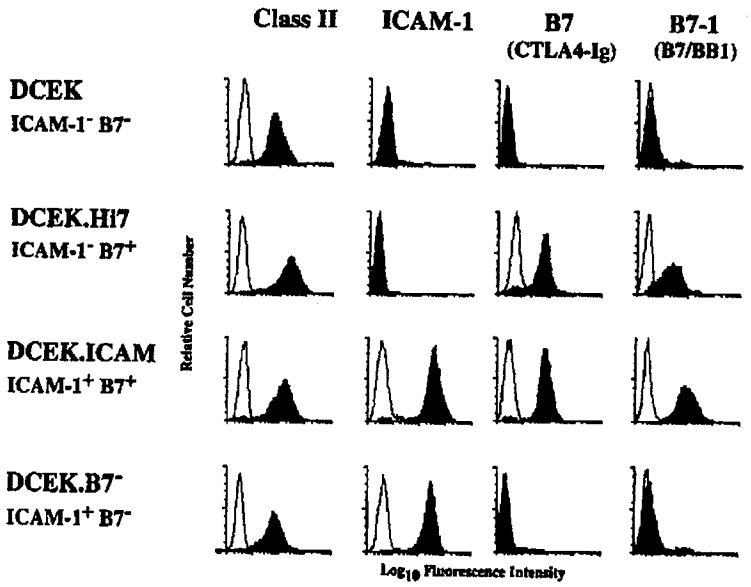

FIGURE 1. Surface marker expression of fibroblast cell lines. Different fibroblast cell lines were stained for class II molecules (M5.114), ICAM-1 (3E2B), B7 (CTLA4-Ig), and B7–1 (1G10) expression as described in *Materials and Methods*. The marker staining is indicated by the closed histograms; background staining with the respective isotype control Ab is shown in open histograms.

at 2 × 10^6/ml 37°C for 2 h, followed by washing. Mitomycin-C (100 μg/ml, Sigma) was added during the last 30 min. For the experiments using anti-CD3 as a stimulus, the APC were only mitomycin treated.

Cell cultures. Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with penicillin, streptomycin, glutamine, 2-ME, HEPES, and 7.5% FCS (HyClone Labs, Logan, UT). Cultures were set up in 200 μl volumes in flat-bottom 96-well plates (Costar, Cambridge, MA). 2.5 × 10^4 high-density $CD4^+$ T cells were stimulated in the presence of varying numbers of APC that had been prepulsed with PCCF for 2 h. For the PCCF titration, 2.5 × 10^4 high-density $CD4^+$ T cells were stimulated in the presence of 1.25 × 10^4 APC and varying doses of PCCF (0.001–10 μM), 10 μM providing optimum stimulation (24, 27). For inhibition studies, blocking mAb (5 μg/ml) were added at the initiation of the culture. For stimulation with anti-CD3, wells were coated with 0.5, 5, or 50 μg/ml 2C11 (50 μl in PBS) for 2 h at 37°C, followed by washing. Anti-CD28 ascites used at varying dilutions or varying numbers of the different cell lines were used as sources of T cell costimulation. Proliferation usually was measured between 48 and 64 h of culture by incorporation of [^{3}H]thymidine (1 μCi/well) during the last 16 h. IL-2 secretion was assessed in supernatants harvested at 36 h as previously described (27) using the NK bioassay, in the presence of anti-IL-4 (11B11).

Results

Characterization of fibroblast cell lines expressing ICAM-1 and B7 (B7–1)

To develop a panel of APC that differed only in costimulatory molecules, we started with a fibroblast line that Dr. R. Germain had transfected with I-E^k and that was known to express B7. We also obtained from Dr. A. Brian a variant that their laboratory had transfected with ICAM-1. These were termed respectively DCEK.Hi7 and DCEK.ICAM. In our laboratory, we selected a B7–1 negative variant of DCEK.ICAM that we call DCEK.$B7^-$. As a control we used DCEK.Hi7 cells sorted to be B7–1 negative, termed DCEK. We analyzed these four fibroblast populations for their expression of a panel of relevant costimulatory molecules and Figure 1 shows the flow cytometry analysis of class II (I-E^k), ICAM-1, and B7 expression. As shown previously by Kuhlman and colleagues (29), both the fibroblast cell lines transfected with I-E^k (DCEK.Hi7) and additionally transfected with ICAM-1 (DCEK.ICAM) expressed equivalent levels of MHC class II molecules, but ICAM-1 was only present on the DCEK.ICAM line. Interestingly, both of these cell lines expressed B7 molecule(s) as assessed by CTLA4-Ig staining. Using Abs now available against two of the B7 molecules (B7–1 and B7–2, Ref. 31), we found that both fibroblast lines expressed B7–1 (see Fig. 1), but not B7–2 (data not shown). However, this does not exclude the presence of another ligand for CTLA-4. The third cell line (DCEK.$B7^-$) was obtained from DCEK.ICAM cells that lost expression of B7, using immunofluorescence and single-cell sorting. These cells were class II^+, ICAM-1^+, and B7 negative, as shown in Figure 1. The loss of B7 was permanent, no expression being seen over several months' passage or after interaction with T cells (C. Dubey, unpublished observations). Finally, the fourth cell population (DCEK) was obtained after sorting out cells from the DCEK.Hi7 cell line, which had spontaneously lost expression of B7. These cells were class II^+, ICAM-1^-, and B7–1^-, as shown in Figure 1. The fibroblast APC did not express any other known costimulatory molecules that we measured as assessed by immunofluorescence including LFA-1, HSA, CD48 (LFA-3), VCAM-1, or VLA-4 (data not shown).

Thus, these fibroblast lines represent unusually homogeneous populations that can be used to functionally dissect the contribution of the B7:CD28/CTLA-4 and ICAM-1:LFA-1 costimulatory pathways in naive CD4 activation. For purposes of clarity, DCEK cells negative for ICAM-1 and positive for B7 (DCEK.Hi7) will be referred to as

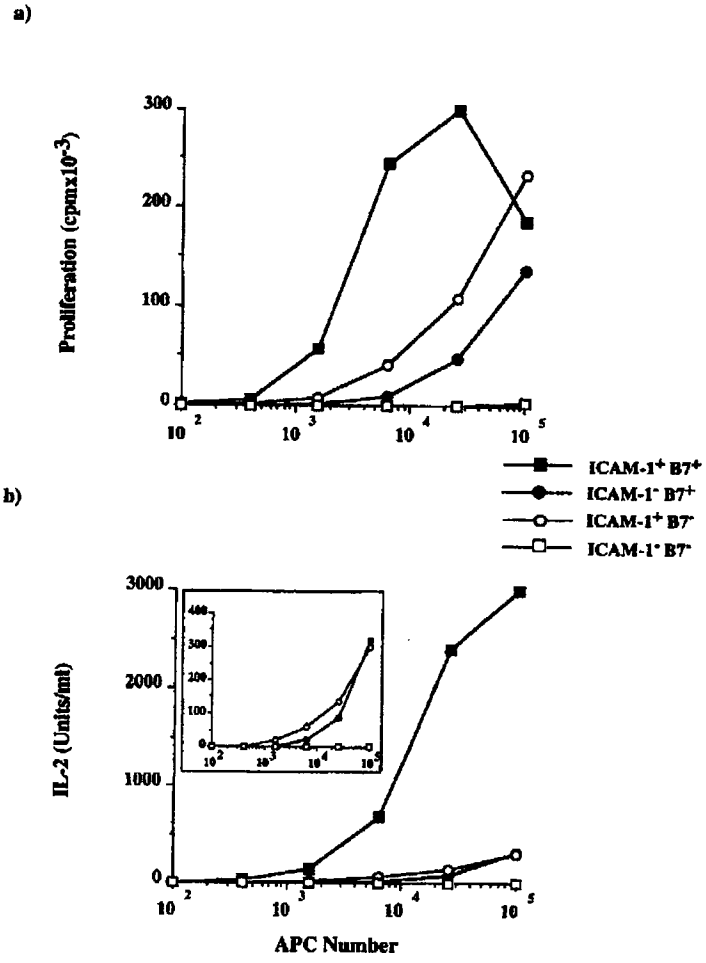

FIGURE 2. Double ICAM-1 and B7-1-expressing fibroblasts are the most efficient stimulators of naive T cell activation. 2.5 × 10^4 Vβ3/Vα11 naive $CD4^+$ T cells were stimulated in the presence of varying numbers of ICAM-1 (○), B7 (●), or ICAM-1 and B7 (■) expressing class II^+ fibroblasts that had been prepulsed with PCCF. A control cell line expressing class II^+ only was added (□). Proliferation (*a*) was assessed between 48 and 64 h and IL-2 secretion (*b*) at 36 h. [^{3}H]Thymidine incorporation in the presence of each APC line but in the absence of PCCF was less than 2000 cpm, and no detectable IL-2 (<10U/ml) was found in either of these culture supernatants. Results show one representative experiment of five.

$ICAM\text{-}1^-$ $B7^+$; DCEK cells positive for B7 and ICAM-1 (DCEK.ICAM) as $ICAM\text{-}1^+$ $B7^+$; DCEK cells positive for ICAM-1 and negative for B7 (DCEK.$B7^-$) as $ICAM\text{-}1^+$ $B7^-$; and DCEK cells negative for ICAM-1 and negative for B7 (DCEK) as $ICAM\text{-}1^-$ $B7^-$.

APC expressing both ICAM-1 and B7-1 induce optimum Ag-specific naive T cell activation

To directly assess the response of naive T cells, we isolated $CD4^+$ T cells from mice transgenic for a TCR (Vβ3/Vα11) specific for a peptide of pigeon cytochrome *c* (PCCF 88–104) expressed on $I\text{-}E^k$ (26). We have previously shown that the majority of the $CD4^+$ T cells from these mice (90 to 95%) express high levels of CD45RB, in association with a pattern of markers characteristic of naive cells, including high levels of L-selectin (Mel-14), and low levels of CD44 (Pgp-1). In addition, these cells are resting and respond to Ag (PCCF) presented on $I\text{-}E^k$ by proliferating and secreting IL-2 (24). They produce only low or undectable levels of other cytokines. The remaining $CD4^+$ T cells (5 to 10%) from these animals have the reciprocal phenotype associated with memory cells ($CD45RB^{low}$, $Mel\text{-}14^{low}$, $Pgp\text{-}1^{high}$). They are also resting cells but do not express the Vβ3/Vα11 TCR and do not respond to PCCF (24). Thus, the contaminating "memory" phenotype cells make no significant contribution to the Ag-specific response.

To investigate which APC costimulatory molecules were optimum for peptide-induced activation, we cultured 2.5 × 10^4 T cells with varying numbers of the four different mitomycin-treated fibroblast cell lines. The APC were pulsed with 20 μM, a saturating dose of peptide-pulsing APC (27), to ensure that signaling via the TCR was not limiting. A representative experiment is shown in Figure 2*a* (T cell proliferation at 48 to 64 h) and Figure 2*b*

(IL-2 secretion). Proliferation (<2000 cpm) and IL-2 secretion (<10 U/ml) in the absence of exogeneous APC or Ag was not seen. APC did not proliferate or produce IL-2 when cultured alone (not shown).

All three Ag-pulsed fibroblast APC expressing ICAM-1, B7–1, or ICAM-1 and B7–1 molecules induced detectable T cell proliferation, but there were dramatic differences between the APC that expressed only B7–1 or ICAM-1 and those expressing both molecules. The APC expressing both ICAM-1 and B7–1 costimulatory molecules were far better stimulators at low cell numbers, initiating a detectable response when less than 2,000 cells were added per culture (1:16 APC:T ratio) and stimulating an optimum response at 25,000 per culture. When APC expressing either ICAM-1 or B7–1 alone were used, responses were detectable only when 8,000 or more cells were present and optimum responses required 100,000 cells (4:1 APC:T ratio). When APC expressing only MHC class II molecules were used, no significant proliferation (<5000 cpm) was observed. In earlier experiments, conventional sources of APC lacking either B7 or expressing low levels of ICAM-1, such as resting I-E^{k+} B cells, stimulated no proliferation or cytokine production by equivalent naive T cells, whereas APC expressing both molecules, such as activated B cells and dendritic cells, were competent as APC (24, 27).

Figure 2*b* shows IL-2 production in similar cultures. IL-2 production showed the same pattern as proliferation but the differences between the APC were even more pronounced. Again, all three APC lines expressing one or two molecules induced detectable IL-2. The response for the single ICAM-1- or B7–1-expressing cells is best seen in the enlarged insert shown on the upper left of the figure. APC expressing either B7–1 or ICAM-1 induced significant amounts of IL-2 (25–60 U/ml) when 8,000 cells were present and maximum IL-2 production (300 U/ml) was reached at a high APC:T ratio (4:1; 100,000 cells). Increasing the APC number further up to 400,000 did not allow the single-expressing APC to induce responses equivalent to the double-expressing cells (data not shown). In contrast, the ICAM-1^+B7^+ cell line not only induced significant IL-2 at a low cell number (148 U/ml with 1,600 cells), but induced massive IL-2 secretion at a higher cell number (2,400 U/ml and 3,000 U/ml with 25,000 and 100,000 cells, respectively). As for proliferation, when APC expressing only MHC class II molecules were used, no IL-2 secretion was detected.

The double-expressing APC were considerably more effective. To generate a response (proliferation and IL-2 production) similar to the optimum response obtained with single-expressing cells, at least 10-fold fewer double-expressing APC cells were needed. Therefore, B7–1 and ICAM-1 coexpression by APC led to strong synergy in the initiation of the response of naive T cells. When the single ICAM-1- and B7–1-expressing APC were mixed together, an additive effect on T cell proliferation and IL-2 production was observed, although the dual-expressing cells were still much better stimulators (data not shown). These results suggest that the most efficient costimulation depends on coexpression of ICAM-1 and B7–1 on the same APC.

Since ICAM-1 is known principally as an adhesion molecule, one interpretation of the results would be that ICAM-1 does not itself provide costimulatory signals, but by increasing intercellular adhesion, merely makes those delivered by other unidentified costimulatory molecules more effective. This is unlikely in our system model, since the control cell line (ICAM-1 and B7–1 negative) did not induce naive T cell activation. This lack of costimulatory capacity was not due to any artifact that may have occurred during the cell sorting, as this same cell line could effectively activate effector CD4 cells (data not shown), which respond somewhat in the absence of costimulus (C. Dubey, unpublished observations, and see Ref. 27). These results rule out the possibility that molecules other than ICAM-1 or B7–1 present on the fibroblast cell line are critically involved in naive T cell activation.

The separate costimulatory functions of ICAM-1 and B7–1 were also confirmed by inhibition studies to assess the relative contributions of ICAM-1:LFA-1 interaction and B7 family interaction (Fig. 3). The fusion protein CTLA4-Ig (28), which interacts with both B7–1 and B7–2, was added to block CD28-mediated signals and anti-LFA-1 mAb to block LFA-1-mediated signals. As in the previous experiment, 2.5×10^4 T cells were cultured with varying numbers of the three different mitomycin-treated fibroblast cell lines expressing one or two molecules. Blocking Abs were added at the initiation of the culture. When the double ICAM-1 and B7–1-expressing fibroblasts were used as APC (upper graphs), addition of either anti-LFA-1 or CTLA4-Ig only partially inhibited the proliferative response and IL-2 production. Simultaneous blockade with both anti-LFA-1 and CTLA4-Ig produced complete inhibition of both T cell proliferation and IL-2 production at low cell numbers of APC (1:4 APC:T ratio). It is likely that at high APC numbers, small responses were still seen due to an excess number of molecules and inefficient Ab blocking. The specificity of the blocking is indicated by the fact that CTLA4-Ig did not affect costimulation (proliferation and IL-2 production) induced by ICAM-1^+ $B7^-$ cells (bottom graphs) (no significant inhibition as compared with control-Ig, not shown), and that anti-LFA-1 mAb did not affect costimulation induced by ICAM-1^- $B7^+$ cells (middle graphs). Moreover, in each case where single-expressing APC were used, only the relevant blocking agent was very effective. CTLA4-Ig inhibited completely the proliferative and IL-2 response induced by ICAM-1^- $B7^+$ cells, except at high cell numbers (100,000 cells), and anti-LFA-1 mAb inhibited the response induced by ICAM-1^+ $B7^-$ cells.

These results suggest that ICAM-1 can act independently of B7 as a costimulatory molecule, since even with

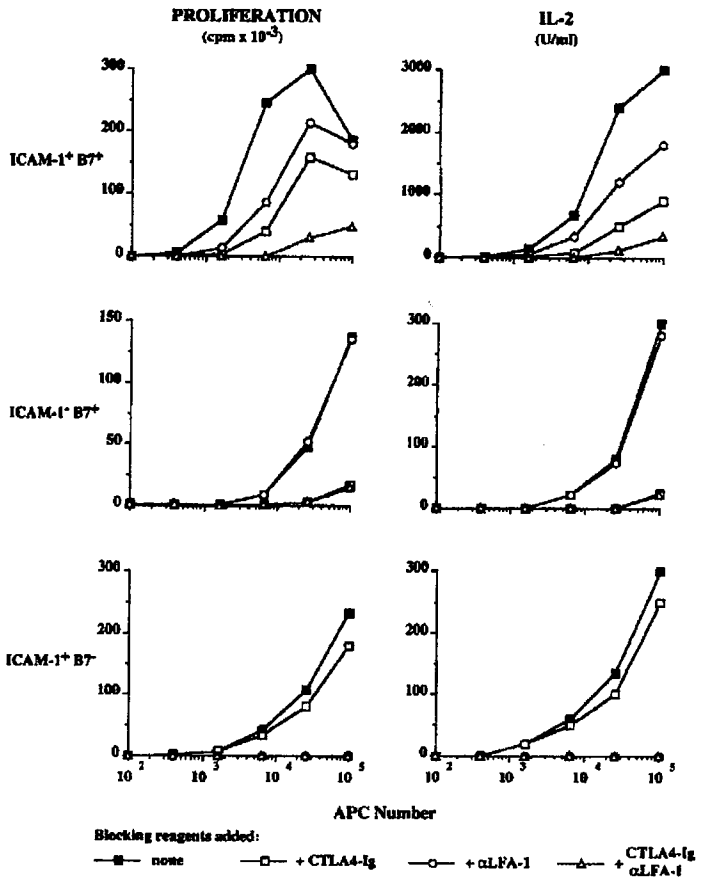

FIGURE 3. B7–1 and ICAM-1 mediate specific costimulatory signals. 2.5×10^4 PCCF-specific naive $CD4^+$ T cells were stimulated in the presence of varying numbers of ICAM-1 (○), B7 (●), or ICAM-1 and B7 (■) expressing class II^+ fibroblasts that had been prepulsed with PCCF. Costimulatory molecule-blocking reagents were added at a final concentration of 5 μg/ml at time 0 hr. Proliferation was assessed between 48 to 64 h and IL-2 secretion at 36 h. Results show one representative experiment of three.

a $B7^-$ APC, a response was obtained and that response was blocked completely by anti-LFA-1. These results, in addition to those obtained with the control cell line in the previous experiment, demonstrate that no molecules expressed by the fibroblasts other than ICAM-1 and B7–1 are able to independently costimulate naive T cell activation, since blocking of the B7–1 and ICAM-1 pathways produced virtually complete inhibition. However, it is likely that providing both ICAM-1 and B7–1 on one APC gives much better stimulation, not only because ICAM-1 and B7–1 are both costimulatory, but also because increased adhesion from these molecules may modulate TCR signaling.

Taken together, these results demonstrate that ICAM-1 and B7–1 synergized in stimulating naive CD4 T cells with peptide Ag, but that either ICAM-1 or B7–1 was sufficient for significant response. B7–1 and ICAM-1 were roughly equivalent in their ability to provide naive T cell costimulation, and the two costimulatory pathways acted independently of one another.

ICAM-1 and B7–1 act synergistically to induce IL-2 production in response to anti-CD3

To confirm the costimulatory role of both ICAM-1 and B7 and to evaluate the effect of the strength of the TCR signal on the relative dependence on costimulation, we used a system with platebound anti-CD3. A concentration of anti-CD3 of 50 μg/ml was used to ensure that signaling via the TCR was not limiting and we compared this to a low density of TCR signaling with 0.5 μg/ml. To avoid any possible stimulation of the putative contaminating memory cell population ($CD45RB^{low}$), naive CD4 cells from transgenic mice were isolated by positive selection on magnetic beads based on CD45RB high expression as described in *Materials and Methods*. Figure 4 shows the responses of naive T cells (2.5×10^4) stimulated with either 50 or 0.5 μg/ml of anti-CD3 in the presence or absence of increasing numbers of the different mitomycin-treated non-Ag-pulsed fibroblast cell lines expressing ICAM-1, B7–1, or both. The results confirm that naive CD4 T cell activation

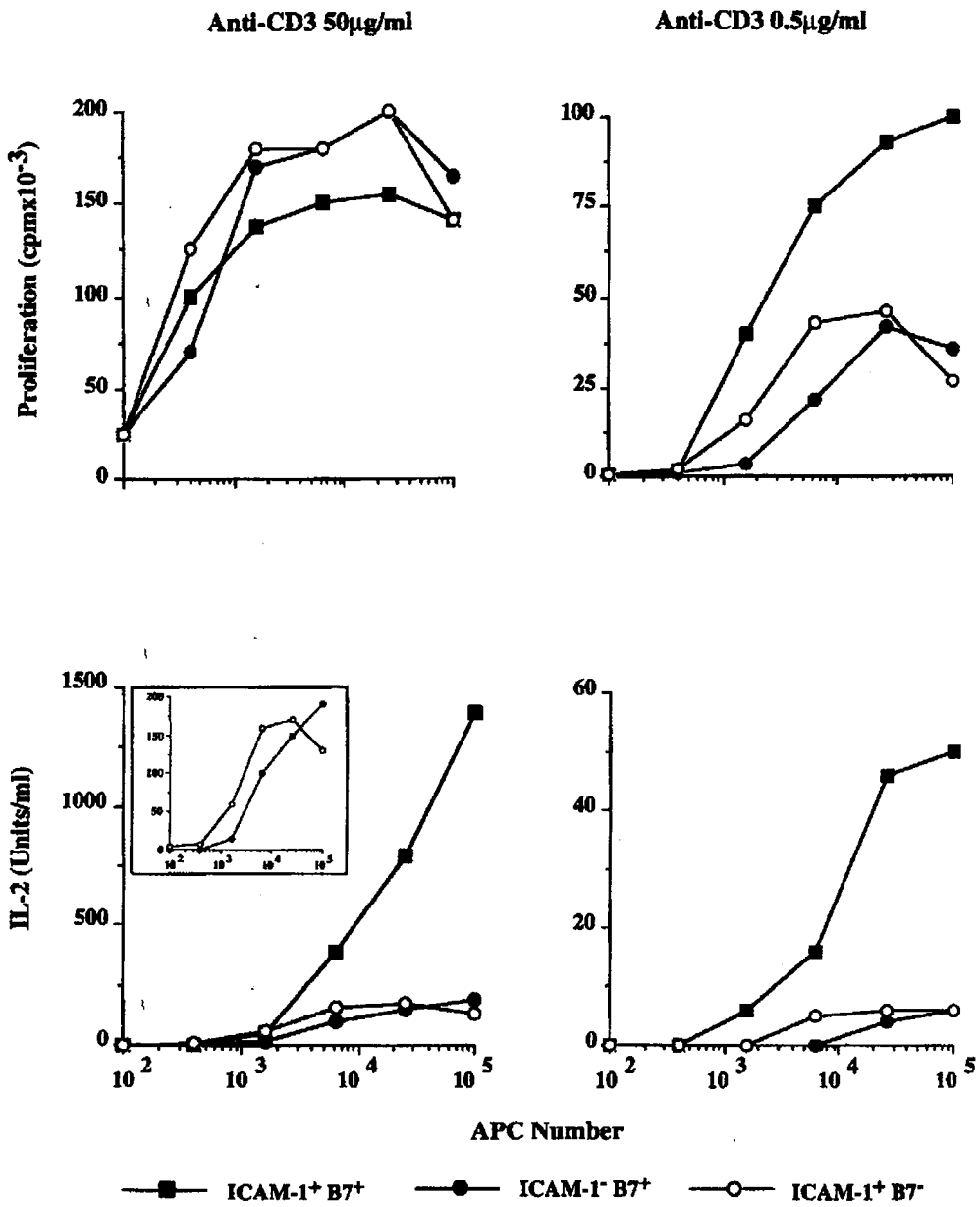

FIGURE 4. Response of naive CD4 cells is critically dependent on costimulatory molecules. ICAM-1 and B7–1-expressing fibroblasts are the most efficient stimulators of naive T cell activation. CD45RB^{high} naive CD4$^+$ T cells were isolated on magnetic beads. 2.5 × 10^4 T cells were stimulated in the presence of varying numbers of ICAM-1 (○), B7 (●), or ICAM-1 and B7 (■) expressing fibroblasts with immobilized anti-CD3 (50 μg and 0.5 μg/ml). Proliferation was assessed between 72 and 88 h and IL-2 secretion at 36 h. T cell response in the absence of accessory cells is shown by the points on the y-axes. Results show one representative experiment of three.

is critically dependent on costimulatory signals. In the absence of added APC (anti-CD3 alone), naive CD4$^+$ T cells proliferated little (anti-CD3 50 μg/ml) or not at all (anti-CD3 0.5 μg/ml) and they produced no detectable quantities of IL-2. Interestingly, unlike the peptide stimulation seen above, induction of a naive T cell proliferation with anti-CD3 at high density was equivalent, whether ICAM-1, B7–1, or both molecule-expressing cells were

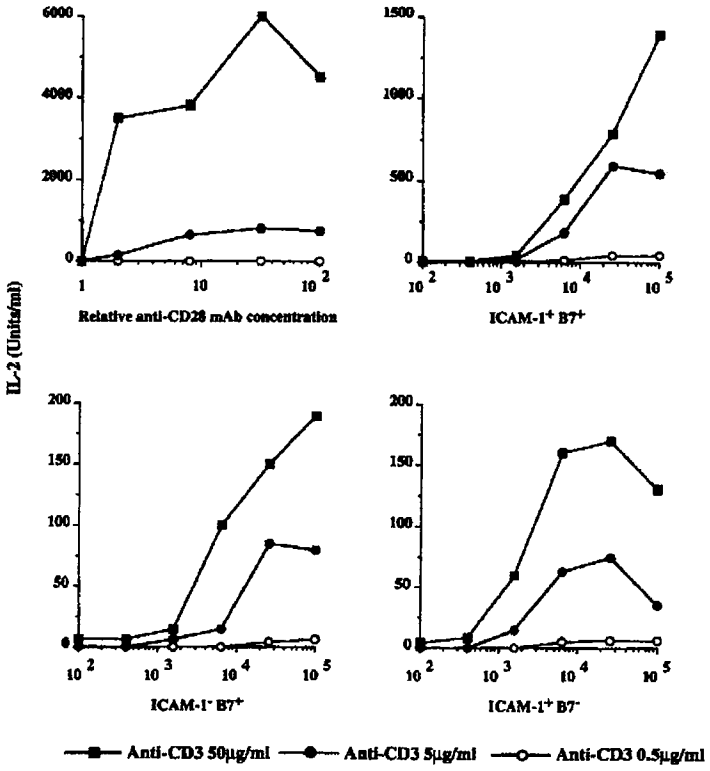

FIGURE 5. Naive requirement for costimuli varies depending on the strength of the TCR signal. 2.5 × 10^4 CD45RB^{high} T cells were stimulated with different concentrations of plate-bound anti-CD3 (0.5 ○, 5 ●, 50 ■ μg/ml). Costimulus was provided by titrating in anti-CD28 or different numbers of the three fibroblast cell lines. IL-2 was measured at 36 h. (Similar curves were seen when assessing CD4 T cell proliferation). T cell response in the absence of costimulus is shown by the points on the *y*-axes. Results show one representative experiment of three.

used as costimuli, over a broad range of doses of fibroblast APC. Increasing the number of fibroblasts clearly provided costimulation, but each of the fibroblast lines was effective. In contrast, at the lower density of TCR signaling, fibroblasts coexpressing ICAM-1 and B7–1 could provide costimulation at the lowest APC numbers to induce naive T cell proliferation, a situation comparable to that seen when Ag was used. In this case, the effects of coexpression showed somewhat weaker synergy than when peptide Ag provided the TCR signal. Somewhat different results were seen for IL-2 production. The responses of naive cells to high-density anti-CD3 were more similar to those with Ag, with IL-2 recovery in the supernatant tremendously enhanced when the dual-expressing cells (ICAM-1$^+$ B7$^+$) were used as costimuli. Parallel results were seen with less anti-CD3, although much less IL-2 was produced when the TCR signal was weak (note the difference in scales of IL-2 production). Thus, for IL-2 production there was excellent B7–1/ICAM-1 synergy at both high and low anti-CD3 doses. The ratio between the maximal IL-2 produced by T cells when costimulated with ICAM-1 plus B7–1 as opposed to ICAM-1 or B7–1 alone was about the same in the two situations (8:1 vs 10:1). The IL-2 production of naive CD4$^+$ T cells to PCCF was greater than to anti-CD3, as reported previously in the same systems using T-depleted spleen cells as APC (27).

Naive requirement for costimulation varies depending on the strength of the TCR signal

To further analyze whether the dependence on costimuli seen above would vary with the strength of TCR signaling, we used several doses of anti-CD3 as a TCR signal. 2.5 × 10^4 naive CD45RB^{high} CD4$^+$ T cells were stimulated with three different concentrations of anti-CD3 (0.5, 5, 50 μg/ml) to mimic a weak, intermediate, or strong density of TCR signaling. Costimulation was provided by titrating in anti-CD28 Ab, or different numbers of the three different mitomycin-treated fibroblast cell lines, and results of effects on IL-2 production are shown in Figure 5. As shown previously, TCR signaling in the absence of costimuli (anti-CD3 alone) did not induce detectable IL-2, even at extremely high levels of TCR ligation (50 μg/ml). Provision of a costimulatory signal in the form of anti-CD28 mAb, which crosslinks CD28 and mimics the effects of B7: CD28 interaction, or in the form of ICAM-1 or B7–1, or ICAM-1 and B7–1-expressing cells, led to increased IL-2 production (Fig. 5) and T cell proliferation (data not shown) in an anti-CD3 dose-dependent manner. The level of IL-2 produced increased with costimulation, but the maximal IL-2 production was seen only with the highest anti-CD3 dose. Moreover, at the higher anti-CD3 doses, lower levels of anti-CD28 or lower number of fibroblasts

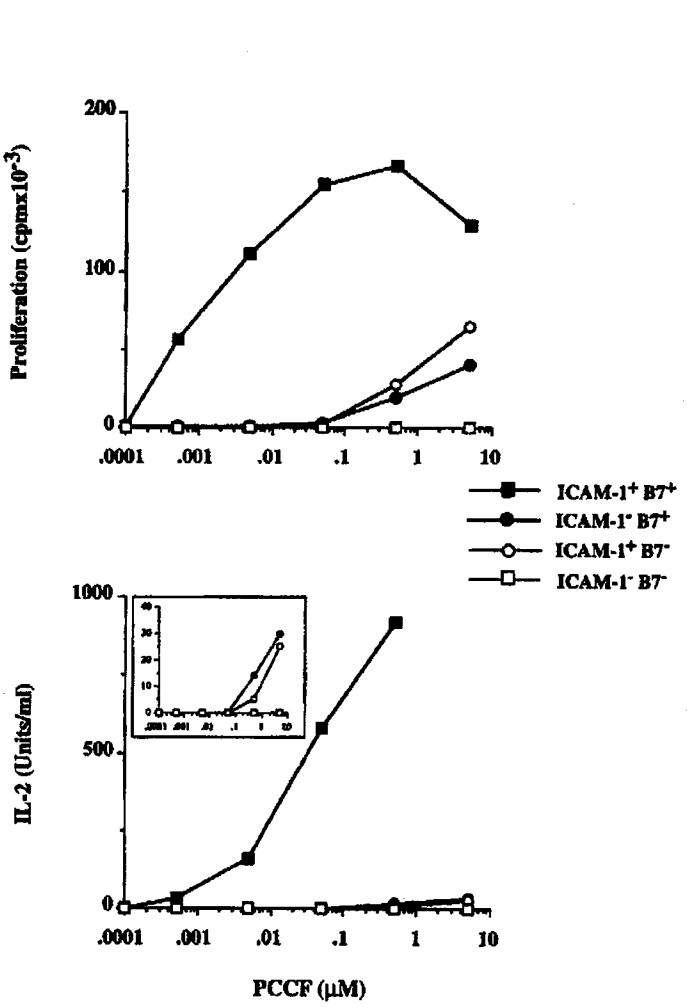

FIGURE 6. Naive requirement for costimuli varies depending on the Ag dose. 2.5 × 10^4 transgenic naive $CD4^+$ T cells were stimulated with different concentrations of PCCF (0.001–10 μM). Costimulus was provided by adding 1.25 × 10^4 ICAM-1 (○), B7 (●), or ICAM-1 and B7 (■) expressing class II^+ fibroblasts. A control cell line expressing class II^+ only was added (□). Proliferation (*a*) was assessed between 48 and 64 h and IL-2 secretion at 36 h. T cell response in the absence of PCCF is shown by the points on the *y*-axes. Results show one representative experiment of two.

expressing costimulatory molecules were needed for optimal IL-2 production. For a low-density TCR signal (0.5 μg/ml), no IL-2 could be detected when anti-CD28 Ab was added, although some proliferation was observed (data not shown). With an intermediate dose (5 μg/ml), high titers of IL-2 were produced (1,000U/ml). At a high dose of anti-CD3 (50 μg/ml), very high titers of IL-2 were secreted (maximum 6,000U/ml) with a very low level of anti-CD28. Each fibroblast provided some costimulation at a high and intermediate anti-CD3 dose, although even at the highest anti-CD3 dose, the single-expressing fibroblasts were much less effective in terms of the levels of IL-2 obtained than were the double-expressing fibroblasts, as seen previously using the peptide Ag (see Fig. 2). Unlike the B7–1 expressing fibroblast, anti-CD28 mAb was able to induce high levels of IL-2 production by naive T cells. This may be due to higher avidity of anti-CD28 mAb for CD28 or to the high density of CD28 crosslinking achieved. As it is difficult to correlate different levels of CD3 cross-linking with antigenic stimulation, a dose-response analysis of peptide concentration is shown in Figure 6. 2.5 × 10^4 T cells were cultured with 1.25 × 10^4 APC expressing ICAM-1, B7–1, or ICAM-1 and B7–1, and varying concentrations of PCCF added to cultures. Proliferation and IL-2 production were measured. Under these conditions, the amount of costimulation provided is constant, with only the TCR signal varying. As seen previously with anti-CD3, maximal IL-2 production and proliferation were seen only with the highest PCCF dose, whatever APCs were used. For a particular dose of Ag, the cell lines expressing both molecules gave the best response, as seen in Figure 2. When APC expressing only MHC class II molecules were used, no response was seen.

The results demonstrate that both the magnitude of the response and the requirement for costimulation depends on the intensity of the TCR signaling, and that activation

is a balance between TCR signaling and costimulatory molecule signaling, with less costimulation required when the TCR signal is strong.

Discussion

We have investigated the effects of two distinct costimulatory interactions on naive CD4 T cell cytokine production and proliferation in response to Ag and to anti-CD3. Using fibroblast "surrogate" APC expressing B7–1, ICAM-1, or both molecules, we find that B7–1 or ICAM-1 individually can provide effective costimulation but that together they synergize to give maximum T cell activation. We also demonstrate that the level of costimulation required depends on the strength of TCR-mediated stimulation, with less costimulation required when TCR signaling was high.

When a naive T cell encounters Ag, several quite distinct outcomes can occur: proliferation, cytokine secretion, and differentiation into effector cells, inactivation, or death or no response to the signal. Which outcome occurs may in most physiologic situations be determined by whether appropriate costimulatory signals are delivered to the responding T cell (32). It is now widely accepted that the B7 molecules (both B7–1 and B7–2) are important costimulatory molecules that interact with the CD28 counterreceptor on resting CD4 T cells and with CTLA-4 and CD28 on activated T cells (10, 22, 33, 34). Particular emphasis has been placed on the CD28:B7 interaction and numerous reports have stressed the importance of CD28 for positive T cell response (see reviews in Refs. 7 and 35). It has also been recently shown by Freeman and colleagues (10), using murine and human B7–1 and B7–2 transfected COS cells, that each molecule can provide costimulatory signals on its own. A recent study of human T cells showed that B7–1 and B7–2 provide similar costimulatory signals for T cell proliferation and cytokine production (36). Our data now indicate that the B7 pathway is strongly costimulatory for naive T cells using either the B7–1 expressing cell line (Figs. 2–6) or anti-CD28 mAb (Fig. 5), and using stimulation with peptide Ag, as well as anti-CD3.

As well as costimulation, another role of cell surface molecules is that of adhesion. Cell adhesion molecules could promote signaling via the TCR by increasing the strength of cell-cell interaction, thus promoting TCR-ligand interaction rather than triggering separate intracellular signal transduction cascades. CD28 triggering is thought to be especially involved in induction of IL-2 (7–10). In contrast, the ICAM-1-integrin interaction has been focused on primarily as an adhesive event (37) that might increase responsiveness by promoting the interaction of TCR with peptide/MHC ligand or other costimulatory molecules with their counterreceptors. However, recent evidence suggests that integrins such as LFA-1 on T cells might transduce signals as well as participate in strengthening the adhesion between Ag-responsive cells and APC. Studies with human T cells stimulated by anti-CD3 as a TCR signal show that biochemically purified ICAM-1 (13), soluble fusion protein of ICAM-1 (Ig chimera of ICAM-1 (14)), or anti-LFA-1 (CD11a) mAb (38) provide a costimulatory signal for resting T cell proliferation and IL-2 production (although IL-2 production was sometimes very low). A recent study from Nadler and colleagues (39), using fibroblasts transfected either with ICAM-1 or B7–1, showed that B7–1 and ICAM-1 were equally potent costimulators of alloreactive T cell proliferation, but that IL-2 production was detectable in the presence of B7–1 only. In the mouse, the data is more indirect for a costimulatory role for ICAM-1:LFA-1 interaction. It has been shown that cotransfection of ICAM-1 and HLA-DR reconstitutes a human Ag-presenting cell function in mouse L cells (40) and inhibition of the ICAM-1:LFA-1 pathway can prolong murine allograft survival (41). Very little is known concerning the precise role of ICAM-1 in Ag-induced responses of naive CD4 T cells. Most studies use resting T cells that include both naive and memory populations and use anti-CD3 or mitogen rather than Ag stimulation. This may give misleading results, as we have shown that memory CD4 T cells have different activation requirements than do naive CD4 T cells, requiring less costimulation for optimum response and giving modest response in the absence of costimulation (25, 27, and C. Dubey and M. Croft, manuscript in preparation).

In our experiments, we have used a transgenic mouse model from which we obtain a homogeneous source of naive Ag-specific CD4 T cells (24). The fact that these cells are not contaminated by activated cells or memory cells is confirmed by the results obtained with anti-CD3 alone, which does not induce any activation of this population (Figs. 4 and 5). The present study using fibroblast cell lines transfected with ICAM-1 as a source of costimulation provides strong evidence that ICAM-1 acts as a classic costimulatory molecule in addition to its adhesion effect. Several lines of evidence support this conclusion. First, the cell line expressing ICAM-1 only ($ICAM\text{-}1^+$ $B7^-$), which expresses no other known costimulators including LFA-1, HSA, CD48, VCAM-1 or VLA-4 (data not shown), is able to induce naive T cell activation (proliferation and IL-2 production) when either Ag or anti-CD3 is used as a TCR signal (Figs. 2–6). The costimulatory ability of the ICAM-1 fibroblast is similar in magnitude and pattern to that observed with the B7–1 only- expressing cell line. Second, when the cells expressing both ICAM-1 and B7–1 are used as costimuli, addition of anti-LFA-1 or CTLA4-Ig to block the LFA-1:ICAM-1 and B7:CD28/CTLA-4 pathways inhibits the response by 50%, whereas simultaneous blockade induces complete inhibition at low cell numbers (Fig. 3). Finally, the cells expressing only class II molecules and neither ICAM-1 nor B7–1 do not activate naive T cells. (Figs. 2, 6). This indicates that in our model no molecules other than

ICAM-1 and B7–1 are effective in inducing T cell response and supports the interpretation that ICAM-1 can costimulate quite effectively in the absence of B7–1 or other costimuli. Thus, it seems likely that ICAM-1 does not act only by enhancing the CD28-B7 pathway when both molecules are coexpressed on the same APC.

ICAM-1, if acting as an adhesion molecule, most likely would function by enhancing the TCR-ligand interaction. It has been estimated that anti-CD3 is at least 1000-fold more avid than interaction of peptide-MHC withTCR (42); therefore, any adhesion effect would be expected to be seen primarily in the case of peptide-mediated CD4 activation when TCR-peptide/MHC avidities are low. It is particularly significant that immobilized anti-CD3, even at a high dose (50 μg/ml), did not induce naive T cell activation (Figs. 4 and 5), but that response was seen if the ICAM-1 expressing fibroblast was added (Figs. 4 and 5). Thus, the fact that ICAM-1 stimulation was observed with immobilized anti-CD3 suggests that this was a costimulatory role rather than increased efficiency of anti-CD3 mAb presentation caused by enhanced adhesion. In the case of Ag presentation, it is likely that both adhesion and costimulation were involved in the responses seen.

Our studies suggest that optimum naive T cell responses require a high level of costimulation only, provided by the combined action of multiple cell surface molecules. A high level of dependence on multiple costimuli explains why some APC, such as dendritic cells and activated B cells (which express many accessory molecules; Refs. 11, 18–20), can activate naive T cells efficiently, while other professional APC, such as macrophages and resting B cells are insufficient (24, 25, 27). Reinforcing the potential importance of ICAM-1 and B7–1 together, we have found that the ICAM-1 and B7–1-expressing cell line (ICAM-1$^+$ B7$^+$) is on a cell-cell basis as good as, if not better than dendritic cells at stimulating naive CD4 T cells (M. Croft, unpublished observations and Ref. 24). In addition, a recent study by Pearson and colleagues (18) demonstrated that in an allogeneic MLR with dendritic cells, as APC, addition of anti-ICAM-1, or CTLA4-Ig, induced a partial inhibition of T cell proliferation, whereas simultaneous blockade produced near complete inhibition. This suggests that the two pathways described here are those used by dendritic cells in their role as activators of T cells. The synergism between costimulatory molecules is highlighted by other studies. In humans, Damle and colleagues (33, 34) suggest that costimulation of resting CD28$^+$ CTLA-4$^-$ T cells by integrins (ICAM-1/LFA-1) induces the surface expression of CTLA-4 on the T cells. This may result in an enhancement of the ability of T cells to respond to B7, as CTLA-4 synergizes with CD28 to augment T cell proliferation. Other costimulators also seem to cooperate in resting T cell activation, for instance, B7 and HSA (43), B7 and ICAM-1 (44, Fig. 2) and within the B7 family between B7–1 and B7–2 (18). Synergy has also been seen between some receptors present on the T cell surface as CD28 and CTLA-4 (33, 34).

Our data also show that the dependence of naive T cells on costimulatory molecules can vary with the strengh of TCR signaling, with much less costimulation required when the TCR stimulus is increased (Figs. 4 and 5). This is a density (or crosslinking) effect, since stimulation with low doses of anti-CD3 (which nonetheless is high affinity) requires high levels of costimulatory signals, whereas high doses result in optimal response with much lower levels of costimulators (Fig. 5). Furthermore, high doses of anti-CD3 or Ag induced a better response (Figs. 5 and 6). This could explain some of the apparent discrepances seen among several reports which analyzed either Ag-, alloantigen-, or polyclonal-induced responses. However, even for a strong TCR signal, the ICAM-1- or B7–1 alone-expressing cell was far less efficient than was the ICAM-1 and B7–1-expressing cell with a weak TCR signal. This suggests that although TCR signal is necessary, in terms of optimal response, costimulation signals are as critical as the TCR signal in enabling response.

An interesting aspect of the data is that, in many instances, substantial proliferative responses were obtained even when little or no IL-2 could be recovered from the medium (Figs. 2, 4, and 6). This is likely to be due to several factors. First, IL-2 may be rapidly consumed during the naive T cell response. Although some consumption is possible, it should be noted that in these studies, supernatants were harvested at 36 h just as the T cell proliferative response was getting underway. This implies that maximal proliferation of naive T cells is achieved even at levels of IL-2 production far below those that can be achieved with good costimulation. The proliferation we assessed was that which occurred between 48 and 64 h. It is thus possible that the higher levels of IL-2 are required to sustain the T cell response for longer periods of time, whereas that initial proliferation is sustained at very low levels of IL-2. Indeed, blocking the IL-2 produced, by adding Abs to IL-2 or IL-2R$_\beta$ chain, almost completely inhibited naive T cell proliferation in response to the peptide Ag, even under conditions where very low levels of IL-2 were detected (data not shown). The high costimulation requirements for peak IL-2 production reinforce the hypothesis that the role of costimulation is primarily to achieve efficient cytokine production rather than to provide other signals required for resting T cell response, such as cell cycle entry, IL-2 receptor expression, and the ability to undergo division in response to IL-2. The separate control of the different aspects of cellular response involved in the expansion of naive T cells and their eventual differentiation into effector and memory populations may suggest the existence of important control points in the overall development of immune responses, whose significance is as yet unappreciated.

Our data, together with that of other investigators, suggest that the two signal model of lymphocyte activation is an oversimplification. The process of naive CD4 T cell

activation is in fact highly complex and involves integration of many signals that determine its outcome. It appears that features of the Ag/TCR interaction (density in particular), cytokine availability, and the relative density of several distinct costimulatory molecule/receptor interactions, which may synergize with each other, are all integrated by the T cell during its activation for cytokine secretion and proliferation. Moreover, our studies support a central costimulatory role, in addition to its adhesive role, for ICAM-1 on APC interacting with LFA-1 on T cells in naive T cell activation and stress the importance of multiple costimulatory signals for efficient response, particularly for optimum IL-2 production.

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Role of CD28-B7 Interactions in Generation and Maintenance of CD8 T Cell Memory

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Although the role of CD28-B7 interaction in the activation of naive T cells is well established, its importance in the generation and maintenance of T cell memory is not well understood. In this study, we examined the requirement for CD28-B7 interactions in primary T cell activation and immune memory. Ag-specific CD8 T cell responses were compared between wild-type (+/+) and CD28-deficient ($CD28^{-/-}$) mice following an acute infection with lymphocytic choriomeningitis virus (LCMV). During the primary response, there was a substantial activation and expansion of LCMV-specific CD8 T cells in both +/+ and $CD28^{-/-}$ mice. However, the magnitude of the primary CD8 T cell response to both dominant and subdominant LCMV CTL epitopes was ~2- to 3-fold lower in $CD28^{-/-}$ mice compared with +/+ mice; the lack of CD28-mediated costimulation did not lead to preferential suppression of CD8 T cell responses to the weaker subdominant epitopes. As seen in $CD28^{-/-}$ mice, blockade of B7-mediated costimulation by CTLA4-Ig treatment of +/+ mice also resulted in a 2-fold reduction in the anti-LCMV CD8 T cell responses. Loss of CD28/B7 interactions did not significantly affect the generation and maintenance of CD8 T cell memory; the magnitude of CD8 T cell memory was ~2-fold lower in $CD28^{-/-}$ mice as compared with +/+ mice. Further, in $CD28^{-/-}$ mice, LCMV-specific memory CD8 T cells showed normal homeostatic proliferation in vivo and also conferred protective immunity. Therefore, CD28 signaling is not necessary for the proliferative renewal and maintenance of memory CD8 T cells. ***The Journal of Immunology*****, 2001, 167: 5565–5573.**

Currently, there is a general consensus that effective activation of naive T cells requires two signals: one dependent on the engagement of the TCR by peptide-MHC complexes, and the second costimulatory signal that is provided by interactions between cell surface molecules on the T cell and the APC (1–4). Numerous studies have indicated that the CD28 molecule, expressed on T cells, provides a potent costimulatory signal following engagement with its ligands, B7-1 and B7-2. Costimulation via CD28 can regulate both proliferation and apoptosis of activated T cells. It has been demonstrated that CD28/B7 interactions are dispensable for initiating early T cell proliferation, but are necessary to sustain late T cell proliferation (5). The lack of late proliferation in the absence of CD28 signaling was associated with increased T cell apoptosis (6). These data suggested that CD28/B7 interactions, in addition to facilitating the initiation of T cell responses, may be important in regulating long-term T cell survival. This notion is supported by studies showing that CD28 ligation increased T cell survival by up-regulating the antiapoptotic gene, $Bcl\text{-}x_L$, and absence of CD28-mediated signaling was associated with low levels of $Bcl\text{-}x_L$ (7, 8). Taken together, these findings establish a precedent that CD28 signaling may play an important role in the survival of memory T cells and in the generation of protective immunity.

Many acute viral infections in humans and mice elicit potent $CD8^+$ T cell responses that are instrumental in clearing virus from the tissues. The requirement for CD28/B7 interactions in eliciting anti-viral $CD8^+$ CTL responses has been examined in several models (9–14). Interactions between CD28 and B7 are obligatory to generate optimal $CD8^+$ CTL responses following influenza and vesicular stomatitis virus (VSV)[4] infections (10, 11). In contrast, CD28/B7 interactions are not essential to elicit primary $CD8^+$ CTL responses following vaccinia virus (VV) and lymphocytic choriomeningitis virus (LCMV) infection (9–10, 12–13). $CD8^+$ T cell responses during viral infections are directed against dominant and subdominant epitopes. It has been reported that CD28 deficiency did not affect the activation of CD8 T cells directed against the dominant CTL epitopes during LCMV infection in mice (9, 13). However, the role of CD28/B7 interactions in activation and expansion of subdominant vs dominant epitope-specific CD8 T cells is not known. Also, the requirement for CD28 signaling in the generation and maintenance of long-term T cell memory is unclear.

Infection of immunocompetent mice with the Armstrong strain of LCMV (LCMV-Arm) elicits a potent CD8 T cell response that eliminates virus within 8–10 days (15–17). The generation of primary anti-LCMV CD8 T cell response is not dependent either on the presence of CD4 T cells (15, 18) or B cells (16). The CD8 T

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[4] Abbreviations used in this paper: VSV, vesicular stomatitis virus; VV, vaccinia virus; LCMV, lymphocytic choriomeningitis virus; LCMV-Arm, the Armstrong CA 1371 strain of LCMV; BrdU, 5-bromo-2′-deoxyuridine; PI, postinfection; NP, nucleoprotein; i.c., intracranial.

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cell response to LCMV is directed against well-characterized dominant and subdominant epitopes (19–20). Furthermore, the techniques of intracellular cytokine staining and MHC class I tetramers allows precise quantitation of $CD8^+$ T cells specific to multiple epitopes of LCMV within the same mouse (9, 13, 20). These features of the LCMV-specific T cell response allowed us to ask the following questions in this study: Is there a differential requirement for costimulation in LCMV-specific CD8 T cell responses against dominant and subdominant CTL epitopes? Is CD28/B7 interaction required for the generation and maintenance of T cell memory? What is the role of CD28/B7 interactions in regulating homeostatic proliferation of memory CD8 T cells? To address these questions, we compared LCMV-specific CD8 T cell responses between wild-type (+/+) and CD28-deficient ($CD28^{-/-}$) mice. Our studies revealed that the generation of primary LCMV-specific CD8 T cell responses (against dominant and subdominant epitopes) does not require CD28 signaling. The generation and maintenance of CD8 T cell memory against both dominant and subdominant epitopes was unperturbed under conditions of CD28 deficiency. Also, loss of CD28/B7 interactions did not affect the homeostatic proliferation of LCMV-specific memory CD8 T cells.

Materials and Methods

Mice

C57BL/6 ($H\text{-}2^b$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The $CD28^{-/-}$ mice (C57BL/6 background) used in these experiments were created by targeted gene disruption, which abrogates surface expression of the CD28 (12). The C57BL/6 LCMV carrier colony was established and bred at Emory University (Atlanta, GA) as described previously (20). Spleen cell preparations from these mice contain LCMV-infected cells that present viral Ag with both MHC class I and II molecules, but lack LCMV-specific T cells.

Virus

LCMV-Arm was used in these studies for infection of mice (21). Mice were infected with LCMV-Arm by i.p. injection (2×10^5 PFU/mouse). To measure protective immunity, LCMV-immune mice were challenged with 10^2 PFU of LCMV-Arm (intracranial (i.c.) infection) or 2×10^6 PFU of LCMV-clone 13 (i.v. injection; 15–17, 21). Infectious virus in serum and tissues was quantitated by plaque assay on Vero cell monolayers as previously described (21).

CTL assay

MHC class I-restricted LCMV-specific CTL activity was determined by ^{51}Cr release assay as previously described (21).

Determination of the LCMV-specific CTLp frequency

CTLp frequency was determined by a limiting dilution assay as described previously (15–17). Spleen cells from LCMV-immunized mice were cultured in graded doses in 96-well flat-bottom plates (12 wells per dose). Syngeneic feeder spleen cells (8×10^5) from uninfected mice and syngeneic stimulator spleen cells (2×10^5) from LCMV carrier mice were irradiated (1200 rad) and added to each well. Recombinant human IL-2 was purchased from BD PharMingen (La Jolla, CA) and was added at a final concentration of 50 U per ml. After 8 days, the contents from each well were split to test CTL activity against LCMV-infected and uninfected MC57 targets in a 6-h ^{51}Cr-release assay.

Flow cytometry

The number of CD8 T cells in the spleen and lymph nodes was determined by staining with specific mAbs followed by FACS analysis, as previously described (16, 20). For FACS analysis, PE-conjugated anti-CD8α (53-6.7), and FITC-conjugated anti-mouse CD44 (IM7) were purchased from BD PharMingen and were used at concentrations recommended by the manufacturer.

Visualization of LCMV-specific CD8 T cells by MHC I tetramer staining

Construction of the MHC I D^b tetramers that contain the LCMV CTL epitope peptides nucleoprotein (NP) 396–404 or gp33–41 has been described previously (20). Spleen cells were surface stained with either FITC- or PE-conjugated anti-CD8 (BD PharMingen) and fluorochrome-labeled MHC I tetramer for 1 h at 4°C. Spleen cells from uninfected mice were always stained in parallel with cells from infected mice as a negative control. To analyze CD28 expression on LCMV-specific memory CD8 T cells, single cell suspensions of splenocytes were stained with anti-CD8, anti-CD28 (BD PharMingen), and MHC I tetramers, followed by three-color flow cytometry.

Quantitation of T cell responses by intracellular staining for IFN-γ

Intracellular staining for IFN-γ in CD8 T cells following stimulation with the specific peptide was done as described previously (20). Spleen cells were incubated in vitro for 5 h at 37°C in medium containing brefeldin A and recombinant human IL-2 (50 U/ml). Cells were either left unstimulated during the culture period or were stimulated with MHC class I-restricted epitope peptides. After the incubation period, the cells were surface stained for CD8 using allophycocyanin- or PE-conjugated Abs and were then stained intracellularly with anti-IFN-γ-FITC using the Cytofix/Cytoperm kit from BD PharMingen. Generally, detection of LCMV-specific CD8 T cells by either intracellular cytokine staining or MHC class I tetramers gives comparable results (20).

Quantitation of virus-specific IFN-γ-secreting T cells

Virus-specific T cell responses were measured by ELISPOT assay as described previously (20). The capture Ab, anti-mouse IFN-γ (clone R4-6A2) and detection Ab, biotinylated anti-mouse IFN-γ (clone XMG1.2) were purchased from BD PharMingen. The ELISPOT plates were purchased from Millipore (Bedford, MA). MHC I-restricted peptides LCMV $NP_{396-404}$, gp33–41, or gp276–286 were used to stimulate CD8 T cells. Uninfected spleen cells contain IFN-γ-producing cells at a frequency of <2 per 10^6 cells with or without stimulation. Quantitation of LCMV-specific CD8 T cells by intracellular cytokine staining or ELISPOT gives comparable results (20).

5-Bromo-2′-deoxyuridine (BrdU) incorporation studies

To monitor proliferation of LCMV-specific memory CD8 T cells in vivo, LCMV-immune mice were given BrdU in drinking water (0.8 mg/ml) for 8 days. After the BrdU pulse, splenocytes were isolated and stained with anti-CD8 and anti-CD44 or fluorochrome-labeled MHC I tetramers. Following surface staining, cells were stained with anti-BrdU Abs, as described previously (20). Flow cytometry and data analysis were performed as described above. Spleen cells from mice that were not exposed to BrdU were always used as negative controls for BrdU staining.

CTLA4-Ig treatment in vivo

The CTLA4-Ig fusion protein used in this study was provided by Bristol-Myers Squibb (New York, NY) and has been described elsewhere (22–24). CTLA4-Ig is a fusion protein that contains the extracellular domain of CTLA4 and is fused to the Cγ region of human Ig. The human Ig control Ab was purchased from BD PharMingen. CTLA4-Ig was injected i.p. at a dose of 200 μg/mouse/injection on days 0, 2, 4, and 6 relative to infection with LCMV-Arm.

Results

Primary CD8 T cell responses in the absence of CD28-B7 interaction

We determined the role of CD28/B7 interactions in generating primary CD8 T cell responses by comparing the CD8 CTL responses between +/+ and $CD28^{-/-}$ mice following an acute infection with LCMV-Arm. On day 8 postinfection (PI), we quantitated the direct ex vivo MHC class I-restricted LCMV-specific CD8 CTL-mediated cytotoxicity in the spleens of +/+ and $CD28^{-/-}$ mice. Consistent with previous reports (10, 12), the LCMV-specific cytotoxic activity in the spleens of $CD28^{-/-}$ mice was comparable with that of +/+ mice (Fig. 1). These data show that generation of LCMV-specific effector CD8 CTL response is not dependent upon CD28/B7 interactions. $CD28^{-/-}$ mice cleared LCMV by day 8 PI similar to +/+ mice (data not shown).

Activated/memory CD8 T cells express elevated levels of CD44 ($CD44^{high}$). The activation and expansion of CD8 T cells following

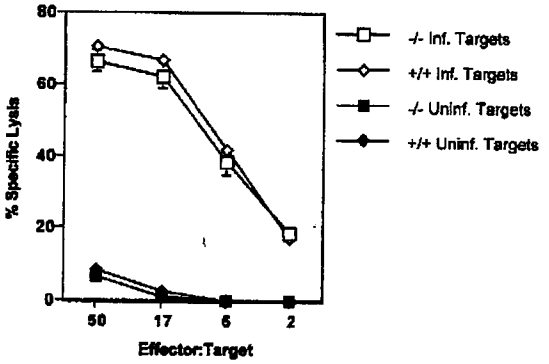

FIGURE 1. LCMV-specific CTL responses in CD28-deficient mice. Spleen cells were isolated from LCMV-infected +/+ and $CD28^{-/-}$ mice on day 8 PI, and MHC class I-restricted CTL activity was measured in a ^{51}Cr release assay using uninfected (Uninf.) and LCMV-infected (Inf.) MC57 cells as targets.

LCMV infection was determined by flow cytometry after staining spleen cells from +/+ and $CD28^{-/-}$ mice for CD8 and CD44. As seen in the +/+ mice, there was a strong activation of $CD8^+$ T cells in the spleens of $CD28^{-/-}$ mice. The total number of activated CD8 T cells in the spleens of $CD28^{-/-}$ mice was ~2-fold lower as compared with +/+ mice (Fig. 2*a*). Uninfected +/+ and $CD28^{-/-}$ mice showed no differences in the total number of naive or activated CD8 T cells (data not shown). We next performed more precise analysis of the activation and expansion of LCMV-specific CD8 T cells using MHC class I tetramers bearing the LCMV NP CTL epitope peptide, $NP_{396-404}$. On the day 8 PI, spleen cells from LCMV-infected +/+ and $CD28^{-/-}$ mice were stained with anti-CD8 Abs and fluorochrome-labeled MHC class I (D^b) tetramers followed by flow cytometric analysis. Representative FACS profiles of staining for $CD8^+$ tetramer-binding cells are shown in Fig. 2*b*. As shown in Fig. 2*b*, the relative proportions of $NP_{396-404}$-specific cells in the spleens of $CD28^{-/-}$ mice was lower compared with +/+ mice. On day 8 PI, the spleens of +/+ and $CD28^{-/-}$ mice had 12–18 × 10^6 and 6–9 × 10^6 $NP_{396-404}$-specific CD8 T cells, respectively. Although high numbers of $NP_{396-404}$-specific CD8 T cells were generated in $CD28^{-/-}$ mice, the overall magnitude was ~2-fold lower compared with +/+ mice. Similar results were obtained following staining with MHC class I tetramers specific to the other LCMV immunodominant epitope in the viral glycoprotein, gp33–41 (data not shown). The effect of B7-CD28 blockade was also examined in +/+ mice treated with CTLA4-Ig. Treatment of +/+ mice with CTLA4-Ig blocks B7 interactions, and this reagent has been shown to block alloantigen-induced T cell responses and certain allograft reactions (22–24). The total number of

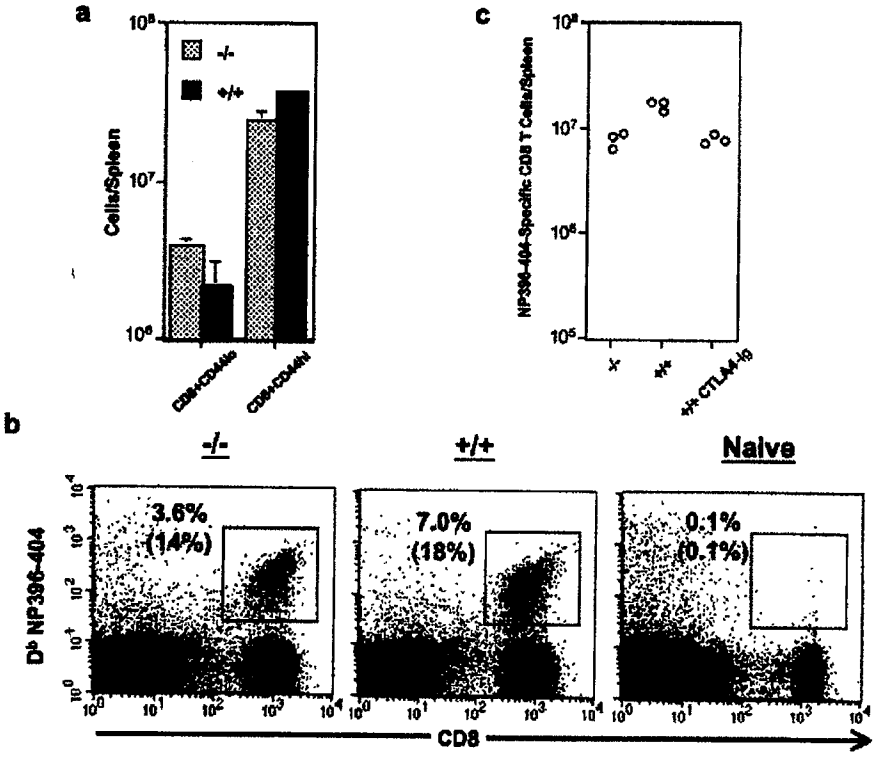

FIGURE 2. Expansion of LCMV-specific $CD8^+$ T cells in $CD28^{-/-}$ mice. *a*, To monitor activation of CD8 T cells, 8 days following infection with LCMV, spleen cells from +/+ and $CD28^{-/-}$ mice were stained with anti-CD8 and anti-CD44 Abs and were analyzed by flow cytometry. Naive CD8 T cells are $CD44^{low}$ and activated/memory CD8 T cells are $CD44^{high}$. *b*, On the eighth day after infection, the number of LCMV-specific CD8 T cells among splenocytes was determined by staining with anti-CD8 Abs and D^b $NP_{396-404}$ tetramers. The flow cytometry plots are gated on spleen cells based on forward and side scatter. The numbers represent percentages of tetramer-binding CD8 T cells among total spleen cells. Numbers in parentheses represent percentages of tetramer-binding cells of total CD8 T cells. *c*, +/+ and −/− mice were infected with LCMV-Arm, and B7-mediated costimulatory interactions in +/+ mice were blocked by treatment with CTLA-4-Ig as described in *Materials and Methods*. On day 8 PI, the total number of $NP_{396-404}$-specific cells in the spleens of LCMV-infected +/+ and −/− mice was determined by staining with D^b $NP_{396-404}$ tetramers.

$NP_{396-404}$-specific CD8 T cells was reduced in +/+ mice treated with CTLA4-Ig to levels similar to $CD28^{-/-}$ mice (Fig. 2*c*).

Role of CD28-B7 interactions in activation of CD8 T cells specific to dominant and subdominant epitopes

The MHC class I-restricted LCMV-specific CD8 T cell epitopes can be divided into dominant and subdominant epitopes. In H-2^b mice, the dominant epitopes are $NP_{396-404}$ and gp33–41, and the subdominant epitopes are gp276–286, $NP_{205-212}$, and gp92–101 (19–20). It was of interest to determine the requirement for CD28-mediated costimulation for optimal activation of CD8 T cells, specific to dominant epitopes vs the weaker subdominant epitopes. The epitope-specific CD8 T cell responses in +/+ and $CD28^{-/-}$ mice were analyzed by intracellular staining for IFN-γ on day 8 PI (Fig. 3). Upon LCMV-Arm infection, $CD28^{-/-}$ mice mounted readily detectable CD8 T cell responses to both dominant and subdominant epitopes. At the peak of immune response, the total number of CD8 T cells specific to all the epitopes was ~2-fold lower in the $CD28^{-/-}$ mice compared with +/+ mice. A previous study has indicated that CD8 T cells specific to the immunodominant epitopes undergo cell division ~15 times during the expansion phase of the anti-LCMV T cell response (20). Therefore, a 2-fold reduction in the number of LCMV-specific CD8 T cells in $CD28^{-/-}$ mice may reflect one less cell division as compared with +/+ mice during the proliferation phase of the CD8 T cell response. In summary, these data suggested that following an acute LCMV infection, the activation of CD8 T cells specific to both dominant and subdominant epitopes is largely independent of CD28-mediated costimulation. Furthermore, these data show that the development of CD8 T cell effector functions, namely cell-mediated cytotoxicity (Fig. 1) and production of IFN-γ (Fig. 3), did not require CD28 signaling.

Generation and maintenance of CD8 T cell memory in the absence of CD28-B7 interactions

Memory CD8 T cells in humans exhibit heterogeneity with respect to CD28 expression; only a subpopulation of memory CD8 T cells in humans express CD28 on their surface (25–29). Although it has been shown that all murine T cells express CD28 constitutively (3), the expression of CD28 on Ag-specific memory CD8 T cells has not been studied. We examined the expression of CD28 on the surface of LCMV-specific memory CD8 T cells by flow cytometry. As illustrated in Fig. 4, LCMV-specific memory CD8 T cells expressed readily detectable levels of CD28 on their surface with

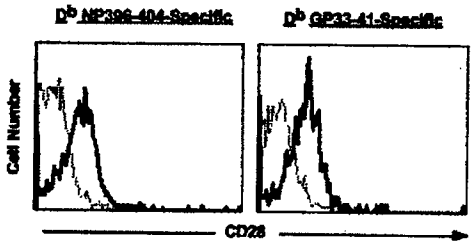

FIGURE 4. CD28 Expression on LCMV-specific memory CD8 T cells. Groups of +/+ and $CD28^{-/-}$ mice were infected with LCMV-Arm. Sixty days following infection, splenocytes were stained with anti-CD8, anti-CD28, and MHC class I tetramers (specific to the LCMV CTL epitopes $NP_{396-404}$ and gp33–41), and were analyzed by flow cytometry. The histograms shown in the figure are gated on tetramer-binding CD8 T cells. The bold and the broken lines in the histograms represent CD28 staining on LCMV-specific memory CD8 T cells from +/+ and $CD28^{-/-}$ mice, respectively. Data are representative of studies using six mice/group.

little or no heterogeneity. Currently, it is not known whether CD28-B7 interactions are necessary to maintain $CD8^+$ T cell memory. To examine the role of CD28-B7 interactions in generating LCMV-specific CD8 T cell memory, $CD28^{-/-}$ mice were immunized with LCMV-Arm, and memory CD8 T cell responses were analyzed by IFN-γ ELISPOT assay at various time points after infection. Fig. 5 shows the kinetics of LCMV-specific CD8 T cell response in +/+ and $CD28^{-/-}$ mice. As shown in Fig. 5, the peak of LCMV-specific CD8 T cell response was attained on day 8 PI, which was followed by a contraction/down-regulatory phase (days 8–15 PI). During the contraction phase of the T cell response, the magnitude of loss of LCMV-specific CD8 T cells in the $CD28^{-/-}$ mice was comparable with that of +/+ mice. Approximately 90% of the LCMV-specific $CD8^+$ T cells were lost (20), presumably by apoptosis (30) during the contraction phase in both +/+ and $CD28^{-/-}$ mice. There is evidence that activation in the absence of CD28-mediated costimulation may lead to T cell apoptosis in vitro (6). However, the data shown in Fig. 5 suggested that in vivo, lack of CD28-mediated costimulation did not lead to an exaggerated death phase of LCMV-specific CD8 T cell response. The contraction phase of the CD8 T cell response is followed by the phase of memory, when a stable pool of memory T cells are maintained indefinitely. Analysis of LCMV-specific CD8 T cell responses revealed that the number of memory cells was

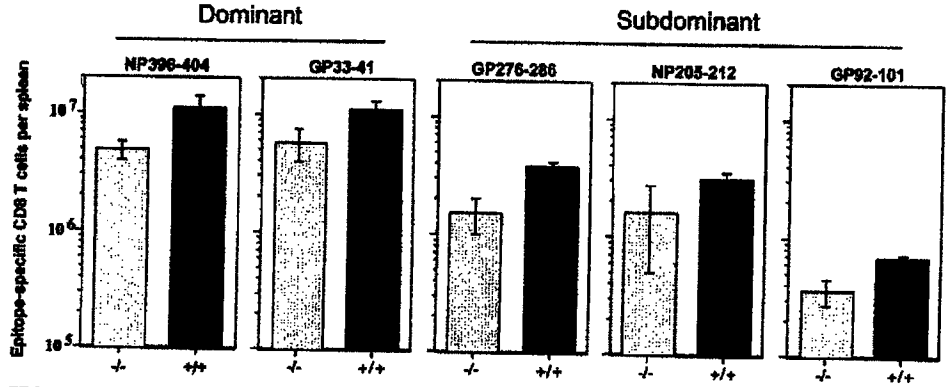

FIGURE 3. CD8 T cell responses to dominant and subdominant epitopes in CD28-deficient mice. On day 8 PI with LCMV-Arm, splenocytes from +/+ and $CD28^{-/-}$ mice were stimulated with CTL epitope peptides, and number of CD8 T cells specific to dominant and subdominant epitopes were determined by intracellular staining for IFN-γ. Data are the means of three to five mice/group.

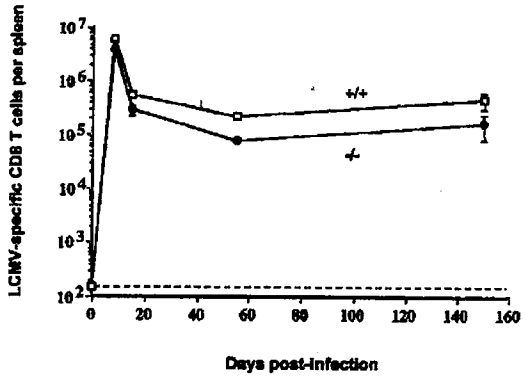

FIGURE 5. CD8 T cell memory in $CD28^{-/-}$ mice. On the indicated days postinfection with LCMV-Arm, the number of LCMV-specific CD8 T cells in the spleens of +/+ and $CD28^{-/-}$ mice were determined by IFN-γ ELISPOT. Spleen cells were stimulated separately with $NP_{396-404}$, gp33–41, and gp276–286 peptides; the total number of IFN-γ-producing cells responding to each of these epitopes was added. The background from unstimulated wells (which was very small) has been subtracted. The dotted line represents the limit of detection of the assays. The error bars represent the SDs. The data are the mean of three to five mice/group at each time point.

~2-fold lower in $CD28^{-/-}$ than in +/+ mice. However, this did not change over time and the lower number of CD8 memory T cells in $CD28^{-/-}$ mice most likely reflected the 2- to 3-fold lower expansion seen in these mice during the acute phase of the CD8 response (day 8). These data were confirmed by quantitating the number of LCMV-specific CTL precursors in the spleens of LCMV-immune +/+ and $CD28^{-/-}$ mice by limiting dilution analysis (data not shown).

We also analyzed CD8 T cell memory in +/+ and $CD28^{-/-}$ mice (292 days PI) using MHC class I tetramers. As shown in Fig. 6*a*, consistent with published data, the spleens of LCMV-immune +/+ mice contained high frequencies of memory CD8 T cells specific to the immunodominant epitopes, $NP_{396-404}$ and gp33–41 (20). LCMV-specific memory CD8 T cells were also readily detected in the spleens of LCMV-immune $CD28^{-/-}$ mice (Fig. 6*a*), and the frequencies were comparable with those in +/+ mice. Memory CD8 T cells specific to dominant and subdominant epitopes were also quantitated in the spleens of LCMV-immune +/+ and $CD28^{-/-}$ mice by staining for intracellular IFN-γ. Data in Fig. 6*b* illustrate that irrespective of epitope specificity, the percentages of memory CD8 T cells in the spleens of LCMV-immune $CD28^{-/-}$ mice were comparable with +/+ mice. Thus, B7-CD28 interaction is not necessary to generate and maintain memory CD8 T cells following an acute LCMV infection.

Activation threshold of LCMV-specific memory CD8 T cells in CD28-deficient mice

We examined the effect of CD28 deficiency on the activation threshold of memory CD8 T cells in LCMV-immune mice. The activation threshold of memory CD8 T cells (specific to both dominant and subdominant epitopes) was compared between LCMV-immune +/+ and $CD28^{-/-}$ mice by measuring IFN-γ production as a function of the concentration of antigenic peptide. As shown in Fig. 7, in both +/+ and $CD28^{-/-}$ mice, the number of IFN-γ-producing LCMV-specific memory CD8 T cells varied in a peptide dose-dependent fashion. As shown in Fig. 7, memory CD8 T cells in $CD28^{-/-}$ mice exhibited a slight difference in the activation threshold at one peptide dilution for three of the four epitopes as compared with +/+ mice; 0.0001 μg/ml for $NP_{396-404}$, 0.001 μg/ml for gp276–286, and 0.0001 μg/ml for NP205–211. Taken together, these data suggest that lack of CD28/B7 interactions did not significantly affect the sensitivity of LCMV-specific memory CD8 T cells to antigenic stimulation in vitro. Further, these data show that memory CD8 T cells generated under conditions of CD28 deficiency may not be qualitatively different as compared with memory CD8 T cells that were generated in the presence of CD28/B7 interactions.

Homeostatic proliferation of LCMV-specific memory CD8 T cells in the absence of CD28/B7 interactions

It is well established that memory T cells undergo homeostatic proliferation, which is believed to be an important mechanism promoting survival by avoiding cell attrition over time. Studies have indicated that cytokines IL-15 and IL-7 may be important for homeostatic proliferation of memory T cells (31–33). Although homeostatic proliferation of memory CD8 T cells is not dependent upon TCR/MHC interactions (34), the role of costimulatory molecules has not been examined. In this study, we determined the requirement for CD28/B7 interactions in the homeostatic proliferation of LCMV-specific memory CD8 T cells. At 60 days PI with LCMV, +/+ and CD28 $^{-/-}$ mice were given BrdU in drinking water for 8 days. At the end of the BrdU pulse, the percentage of $BrdU^+$ cells among LCMV-specific memory CD8 T cells was determined by flow cytometry. As shown in Fig. 8, 23% and 20% of $CD8^+CD44^{high}$ T cells (activated/memory) incorporated BrdU in +/+ and $CD28^{-/-}$ mice, respectively. About 1.5–2% of naive CD8 T cells ($CD44^{low}$) in both +/+ and $CD28^{-/-}$ mice incorporated BrdU over a period of 8 days (data not shown). Importantly, the percentages of $BrdU^+$ memory CD8 T cells specific to two different LCMV CD8 CTL epitopes were comparable between +/+ and $CD28^{-/-}$ mice. Further, blocking interactions between B7 and CD28/CTLA-4 molecules using CTLA-4-Ig fusion proteins did not affect the homeostatic proliferation of LCMV-specific memory CD8 T cells (data not shown). Taken together, these data suggest that CD28/B7 interactions are not obligatory for homeostatic proliferation of memory CD8 T cells. Also, the proliferation rate of memory CD8 T cells was not affected by antigenic specificity: within the same mouse, memory CD8 T cells specific to two different epitopes had similar proliferation rates in both +/+ and $CD28^{-/-}$ mice.

Protective immunity in CD28-deficient mice

T cell memory to LCMV infection is characterized by the ability of memory $CD8^+$ T cells to generate an accelerated response upon re-exposure, leading to viral clearance more rapidly than they do during the primary exposure (17, 20). We examined protective immunity in $CD28^{-/-}$ mice using two challenge models: 1) protection against a lethal i.c. infection and 2) protection against a persistent LCMV infection. Groups of +/+ and $CD28^{-/-}$ mice were immunized by i.p. infection with LCMV-Arm. To evaluate protection against lethal choriomeningitis, 35 days after immunization, these mice were challenged with LCMV-Arm by i.c. injection. Naive +/+ and $CD28^{-/-}$ mice were infected with LCMV-Arm (i.c.) as controls. As shown in Fig. 9*a*, all of the naive +/+ and $CD28^{-/-}$ mice succumbed to an i.c. LCMV-Arm infection by 7–8 days postchallenge. In striking contrast, all of the LCMV-immune +/+ and $CD28^{-/-}$ mice were completely protected against a lethal LCMV challenge, and survived at least up to 4 mo. The clone 13 strain of LCMV establishes persistent infections in naive immunocompetent mice, which is characterized by low CTL responses and disseminated infection of several tissues (15, 21). However, wild-type mice that have recovered from an

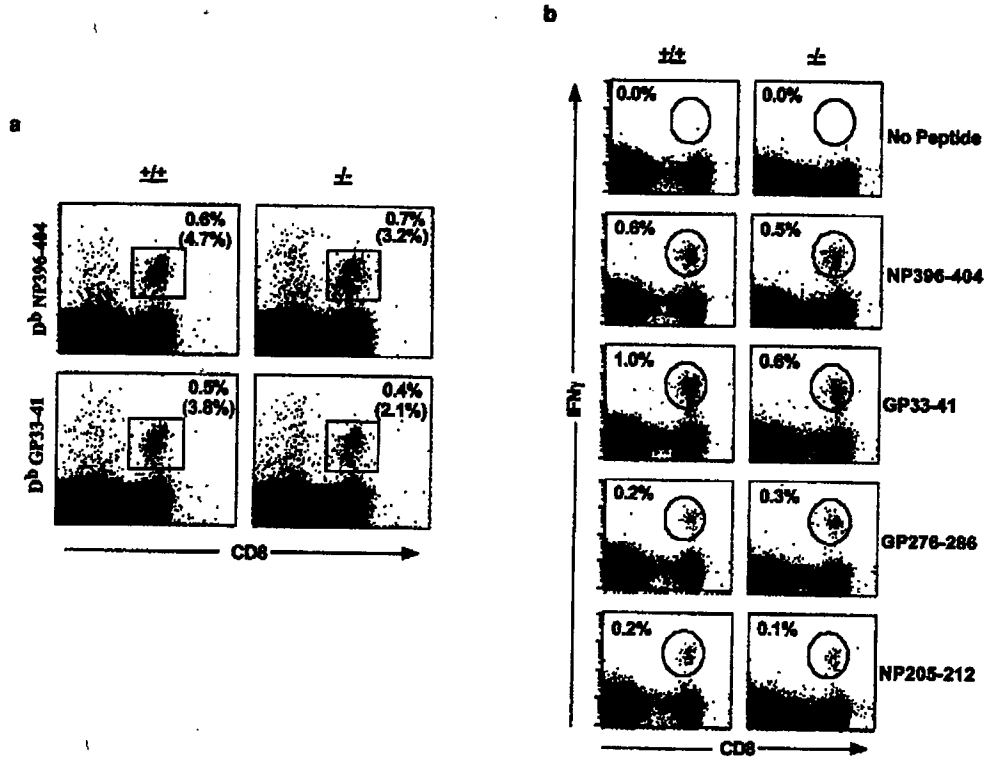

FIGURE 6. Analysis of CD8 T cell memory in CD28$^{-/-}$ mice using MHC I tetramers and intracellular cytokine staining. *a*, Two hundred and ninety-two days following infection with LCMV-Arm, LCMV-specific memory CD8 T cells in the spleens of +/+ and CD28$^{-/-}$ mice were quantitated by staining with MHC I tetramers. The flow cytometry profiles are gated on total splenocytes based on forward and side scatter properties. The numbers represent percentages of tetramer-binding CD8 T cells among splenocytes. Numbers in parentheses represents percentages of tetramer-binding CD8 T cells of total CD8 T cells in the spleen. *b*, Two hundred and ninety-two days following infection with LCMV-Arm, LCMV-specific memory CD8 T cells in the spleens of +/+ and −/− mice were quantitated by intracellular staining for IFN-γ as described in *Materials and Methods*. The numbers represent percentages of IFN-γ-producing CD8 T cells among splenocytes.

acute infection with LCMV-Arm are protected against a persistent infection with LCMV-clone 13 (17). To examine protective immunity against a persistent LCMV infection, +/+ and CD28$^{-/-}$ mice that were previously immunized with LCMV-Arm (100 days PI) were challenged with 2×10^6 PFU LCMV-clone 13 by i.v. injection. Naive +/+ and CD28$^{-/-}$ were also infected with LCMV-clone 13 for comparison. LCMV titer in the serum was determined 5 days after challenge with LCMV clone-13. Data in Fig. 9*b* show that both +/+ and CD28$^{-/-}$ LCMV-immune mice had undetectable levels of infectious virus in the serum; serum from naive +/+ and CD28$^{-/-}$ mice infected with LCMV-clone 13 contained high levels of infectious virus. In summary, these

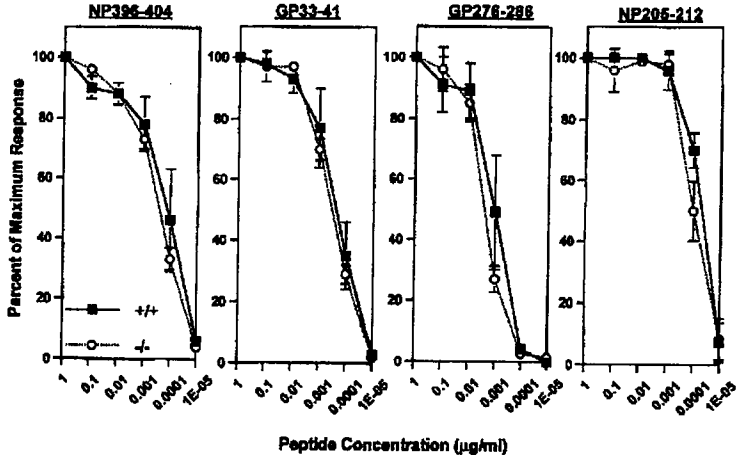

FIGURE 7. Activation threshold of LCMV-specific memory CD8 T cells in CD28$^{-/-}$ mice. Sixty days after infection with LCMV-Arm, the activation threshold of memory CD8 T cells was analyzed by intracellular staining for IFN-γ following stimulation with various LCMV CTL epitope peptides at the indicated concentrations. The results are expressed as a percentage of maximum responsc attained at a peptide concentration (saturating) of 1.0 μg/ml. Data are the means of three mice/group and represents one of two independent experiments.

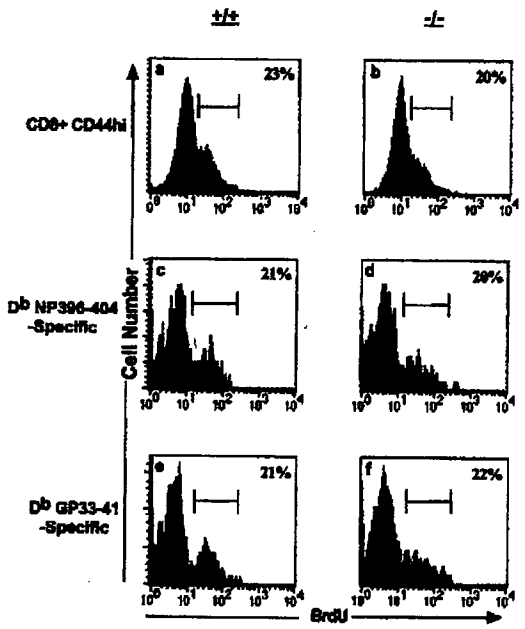

FIGURE 8. Homeostatic proliferation of memory CD8 T cells. Sixty days following infection with LCMV-Arm, +/+ and CD28$^{-/-}$ mice were given BrdU in drinking water for 8 days. At the end of BrdU pulse, spleen cells were stained with anti-CD8, anti-CD44, and anti-BrdU or anti-CD8, fluorochrome-labeled MHC class I tetramers, and anti-BrdU Abs, followed by three-color flow cytometry. *a* and *b*, Gated on CD8^{+}CD44^{high} cells. *c–f*, Gated on CD8^{+} MHC class I tetramer-binding cells. The numbers represent percentages of BrdU^{+} cells among the gated cell population. The data are representative of analysis on four mice/group.

data provide convincing evidence that memory CD8 T cells in LCMV-immune CD28$^{-/-}$ mice confer protective immunity in both peripheral (i.c. infection with LCMV-Arm) and systemic (i.v. infection with LCMV-clone 13) challenge experiments.

Discussion

In this study, we examined the requirement for CD28-mediated costimulation in the activation and expansion of Ag-specific CD8 T cells and maintenance of T cell memory during an acute viral infection. We show that the activation and expansion of LCMV-specific CD8 T cells to both dominant and subdominant epitopes is largely independent of CD28-mediated costimulation. We also show that maintenance of LCMV-specific CD8 T cell memory and protective immunity does not require CD28-B7 interactions.

Previous studies have documented that CD28-B7 interactions are not essential for generating primary LCMV-specific CTL responses (9–13). These studies primarily examined CD8 T cell responses against dominant epitopes of LCMV. The present study confirms and extends these findings. It is well established that CD8 T cell responses during an acute LCMV infection are directed against dominant and subdominant epitopes (19, 20). It is believed that the strength of TCR signaling determines the requirement for costimulation during T cell activation (3, 35). Dominant epitopes most likely are presented at higher densities on the cell surface, thereby delivering a potent signal through the TCR, obviating a need for costimulation. In contrast, activation of T cells by weaker subdominant epitopes may be costimulation dependent. To address this issue, we compared the CD8 T cell responses to dominant and subdominant LCMV CTL epitopes for their dependence on CD28-mediated costimulation. Interestingly, CD28$^{-/-}$ mice generated high numbers of CD8 T cells specific to both dominant and subdominant epitopes, albeit 2-fold lower in magnitude compared with +/+ mice (Fig. 3). Furthermore, the hierarchy of immunodominance among LCMV CTL epitopes was not altered in the absence of CD28-B7 interactions. Although the mechanistic basis of LCMV CTL epitope hierarchy is not known, these data suggested that CD8 T cell responses against dominant and subdominant epitopes are largely independent of CD28-mediated costimulation. These data are in agreement with the studies done by Tan et al. (36), who blocked CD28/CTLA-4-B7 interactions by treating mice with CTLA-4-Ig fusion proteins during an acute LCMV infection. Similar to LCMV infection, CTL responses to VV are not

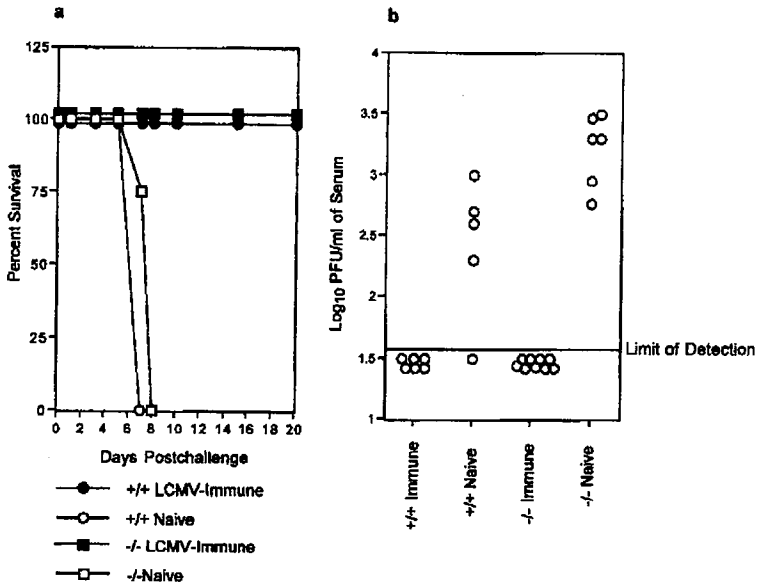

FIGURE 9. Protective immunity in CD28$^{-/-}$ mice. *a*, Thirty-five days after acute LCMV infection, groups of naive and LCMV-immune +/+ and CD28$^{-/-}$ mice were challenged with LCMV by i.c. infection (n = 4–6 mice/group). Following i.c. infection, mice were monitored for mortality. Note that all of the LCMV-immune mice survived, whereas all of the naive mice died by 8 days after i.c. LCMV challenge. *b*, One hundred days after an acute infection with LCMV-Arm, groups of +/+ and CD28$^{-/-}$ mice were challenged with 2 × 10^{6} PFU of LCMV-clone 13 by i.v. injection. Five days after challenge, infectious LCMV in the serum was quantitated by plaque assay on Vero cells. Each point represents the serum virus titer of an individual mouse.

significantly altered by abrogation of CD28 signaling (10). However, B7-CD28 interactions are essential for generating effector CD8 CTL following VSV and influenza virus infection in mice (10, 11). In contrast to LCMV and VV, which replicate efficiently in the lymphoid system, the replication of influenza virus and VSV is very limited in lymphoid tissues. Thus, one common theme that has emerged from these studies is that the CD28-mediated costimulation seems to be dispensable for CTL responses against viruses that replicate to very high levels in the lymphoid system. The rules defining the costimulatory requirements of CD8 T cells may also depend on whether the infection is localized or systemic, and the ability of virus to infect APC, particularly the dendritic cells. Primary CD8 T cell responses to LCMV were normal in the absence of CD40-CD40L interactions (9, 37, 38). In the absence of 41BB-41BBL interactions, LCMV-specific CD8 T cell responses were reduced by ~2-fold (36). It remains to be determined whether CD40-CD40L- and 41BB-41BBL-mediated costimulatory interactions play redundant and/or compensatory roles in activating CD8 T cells under conditions of CD28 deficiency.

The resolution of an acute LCMV infection is dependent upon $CD8^+$ CTLs (39–40) and does not require CD4 T cells (15, 18). Nonetheless, mice acutely infected with LCMV mount a strong CD4 T cell response and develop humoral immunity (41, 42). In contrast to the development of a potent CD8 T cell response, the induction of LCMV-specific CD4 T cell response was compromised in $CD28^{-/-}$ mice (Ref. 13 and M. Suresh, J. K. Whitmire, J. D. Altman, and R. Ahmed, manuscript in preparation). It is likely that lack of CD4 T cell activation in $CD28^{-/-}$ mice reduced the CD8 T cell response by one-half compared with +/+ mice. CD4-deficient mice also exhibit a similar phenotype: the magnitude of LCMV-specific CD8 T cell response was ~2-fold lower in comparison with what was generated in +/+ mice.

In addition to providing costimulatory signals necessary for activation of naive T cells, CD28-B7 interaction has also been shown to enhance survival of activated T cells by inducing the expression of the antiapoptotic gene *Bcl-x_L* (6, 7). Furthermore, *Bcl-x_L* induction prevented Fas- and anti-CD3-induced apoptosis in activated T cells (7, 8). These data suggested that CD28-mediated signaling may be important in the survival of memory T cells. It was of interest to examine the role of CD28-B7 interactions in the generation and maintenance of LCMV-specific memory CD8 T cells. In this study, the maintenance of LCMV-specific CD8 T cell memory was unaffected by lack of CD28/B7 interactions (Figs. 5 and 6). The initial expansion of CD8 T cells during the primary response (clonal burst size) has been shown to be one of the determinants of the magnitude of T cell memory (20). Therefore, a slight reduction in the total number of LCMV-specific memory CD8 T cells in $CD28^{-/-}$ mice most likely reflect a ~2-fold lower expansion of virus-specific CD8 T cells during the primary response.

Studies have shown that Ag dose, duration of TCR stimulation, and number of TCRs engaged can determine the requirement for costimulation during T cell activation. During activation of naive T cells, CD28-mediated costimulation reduces the number of TCRs that need to be triggered by the Ag (43). It was of interest to examine the activation threshold of memory CD8 T cells in the absence of CD28/B7 interactions. To this end, we compared the activation thresholds of memory CD8 T cells specific to multiple epitopes between +/+ and $CD28^{-/-}$ mice. These experiments revealed that loss of CD28/B7 interactions did not significantly affect the activation thresholds of memory CD8 T cells (Fig. 7). One intriguing finding was that the activation threshold of memory CD8 T cells specific to the subdominant epitope $NP_{205-212}$ was lower as compared with memory CD8 T cells specific to the dominant epitopes $NP_{396-404}$ and gp33–41 (even in the presence of CD28/B7 interactions in +/+ mice). The increased sensitivity of $NP_{205-212}$-specific memory CD8 T cells to peptide stimulation cannot be explained based on the differences in the binding affinities of peptides to the MHC I molecule. This is because the MHC-binding affinity of peptide $NP_{396-404}$ is substantially greater than for $NP_{205-212}$ (19). One possibility is that during the primary response, low-level presentation of $NP_{205-212}$ subdominant epitope by the APCs may selectively activate CD8 T cells with high affinity. Alternatively, the repertoire of CD8 T cells that recognize $NP_{205-212}$ are inherently of high affinity and/or avidity. Nevertheless, taken together, these data suggested that CD28-deficient memory CD8 T cells may be qualitatively similar to +/+ memory CD8 T cells.

According to the current axiom, maintenance of memory T cells is dependent upon homeostatic proliferation, which prevents T cell attrition over time (31–33). We examined whether homeostatic proliferation of LCMV-specific memory CD8 T cells is affected in the absence of CD28-B7 interactions. In vivo BrdU labeling studies indicated that the rate of homeostatic proliferation of LCMV-specific memory CD8 T cells in LCMV-immune $CD28^{-/-}$ mice was comparable with that of immune +/+ mice. These data are consistent with normal maintenance of CD8 T cell memory in $CD28^{-/-}$ mice.

Protective immunity is a definitive marker of T cell memory. +/+ mice that have recovered from an acute LCMV infection are protected against lethal choriomeningitis resulting from an i.c. LCMV challenge. Akin to +/+ mice, memory CD8 T cells in LCMV-immune $CD28^{-/-}$ mice successfully protected against a lethal i.c. infection with LCMV (Fig. 9*a*). LCMV-clone 13 is a highly virulent strain of LCMV that establishes persistent infections in immunocompetent mice (21). However, accelerated CD8 T cell responses in LCMV-immune mice promptly controls LCMV-clone 13 infection, preventing viral persistence (17). LCMV-immune $CD28^{-/-}$ mice were completely protected against a persistent infection with LCMV-clone 13, presumably due to memory CD8 T cell-dependent accelerated viral clearance (Fig. 9*b*). In summary, generation and maintenance of CD8 T cell memory, as assessed by quantitation of virus-specific CD8 T cells by phenotypic and functional assays and protective immunity, is not dependent on CD28/B7 interactions.

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The central role of T cells in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory syndromes. As such, RA is often considered the prototype disease for defining both the molecular and pathological basis of immune-mediated chronic inflammatory disease, and for validating targeted therapies.

The immunogenetics of RA suggest a key role for aberrant pathways of T-cell activation in the initiation and/or perpetuation of disease. In the T-cell activation process, CD4+ T-cells are engaged by antigenic peptide fragments in a complex with HLA class II molecules, in addition to co-stimulatory molecules, such as CD80/CD86, expressed on the surface of professional antigen presenting cells. The strongest evidence supporting a role for CD4+ T cells in disease pathogenesis is the association between RA and HLA-DRB1; however, the functional role of this association has yet to be defined. Susceptibility to RA may also be linked with several RA-associated allelic variants of genes, especially PTPN22, but also CTLA4, IL2RA, IL-2RB, STAT4, PTPN2 and PADI4, many of which encode molecules directly implicated in pathways of T-cell activation.

The presence of inflammatory infiltrates, such as follicular structures, in the synovial membrane provides compelling evidence of ongoing immune reactions in moderate to severe RA. These structures likely play a key role in T cell – B cell cooperation and the local generation of specific autoantibodies; as such, chronically activated synovial T cells represent key cellular targets for therapy. Evidence also supports a role for T-helper (Th) cells, Th17 cells, and impaired CD4+CD25hi regulatory T cell (Treg) function in the pathogenesis of RA.

In addition to discussing a range of issues regarding T-cell activation in RA, this review describes how therapeutic modulation of T-cell function, as opposed to profound immunosuppression or immunodepletion, has been associated with better disease outcomes in clinical trials. Ultimately, elucidation of the distinct effects of co-stimulation modulation with abatacept on T cells should provide key insights into understanding how to restore immune homeostasis in patients with RA.

Introduction

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory syndromes, and has become the prototype disease for defining the molecular and pathological basis of immune-mediated chronic inflammatory disease, as well as for validating targeted therapies. Rheumatoid arthritis targets the synovial lining of joints, bursae and tendon sheaths. At least in the early phase, RA is distinct from other organ-specific autoimmune diseases in that, rather than causing cell death and tissue destruction at the outset, the disease is characterized by the activation and proliferation of stromal tissues in the target organ. Furthermore, no antigen or antigens have been defined in RA that reflect the specific target organ. Given the strong genetic associations between RA and genes encoded within the major histocompatibility complex (MHC), a major challenge over many decades has been to understand how aberrant MHC class II restricted T-cell responses might provoke persistent immuno-inflammatory responses that target the synovial joint. Recent advances in genetics, together with data acquired from animal models of autoimmune arthritis, have provided important new clues.

The immunogenetics of rheumatoid arthritis

In spite of heritability estimates of up to 60% (1), the identification of allelic variants that underpin RA disease pathogenesis has been hindered by the fact that RA susceptibility genes

confer low to moderate risk and have low penetrance (2). The only exception to this is the MHC, for which five genome-wide linkage scans of multiplex families have unambiguously established an important contribution, and allelic variation at the HLA-DRB1 locus remains the dominant, although not exclusive, MHC locus contributing to disease susceptibility (2). Nevertheless, a contemporary approach to understanding the biology of complex polygenic traits proposes that disease-associated gene polymorphism is pathway specific rather than disease specific. This notion is supported by the fact that susceptibility genes impose subtle phenotypic variation in the function of biologic pathways shared between autoimmune syndromes (3).

The most comprehensively studied pathway is undoubtedly T-cell activation. In this activation process $CD4^+$ T-cell clones, expressing rearranged antigen T-cell receptor (TCR) α and β chains, recognize and are engaged by antigenic peptide fragments in a complex with HLA class II molecules and co-stimulatory molecules, such as CD80/CD86, expressed on the surface of professional antigen-presenting cells (APCs). The association between RA and HLA-DRB1, which encodes the polymorphic HLA class II DRβ chain where the greatest variation is confined to a stretch of the DRβ chain alpha helix (residues 67–74) known as the shared epitope (SE) (4), remains perhaps the strongest evidence for a role of $CD4^+$ T cells in disease pathogenesis. However, the functional basis for this association remains the subject of some debate. The most obvious functional impact of HLA-DRB1 polymorphism would be to influence TCR recognition, either through the selection of distinct peptide epitopes for presentation to T cells or through direct effects on MHC/TCR contact residues, altering MHC/TCR avidity during thymic development and the activation of T cells in the periphery (5, 6). Other studies have proposed a link between particular subtypes of HLA-DR4 and the replicative history, based on the erosion of telomeres in the hematopoietic compartment (7, 8). It is thought that this might arise from the expansion of self-reactive T-cell clones through homeostatic proliferation in both naïve and memory T-cell compartments and subsequent contraction of the T-cell repertoire documented in patients with RA (9). Why this should be associated with HLA-DR4 and not other HLA-DR molecules is unclear.

More recent studies propose that RA-associated HLA-DR$\alpha\beta$ molecules may promote immune responses to modified self, including citrullinated proteins (10), that appear to be influenced by environmental factors such as chronic exposure to tobacco smoke (11). According to this model, different subsets of RA-associated HLA-DR molecules might present a distinct profile of citrullinated autoantigens to T cells. The finding of an association between gain-of-expression variants of *PADI4*, which encodes one of several peptidylarginine deiminase (PAD) enzymes that citrullinates proteins, and disease in Japanese, US and European RA populations is, therefore, of particular interest (12, 13). Emerging data further support the notion that RA-associated allelic variants may impose subtle phenotypic effects on pathways of T-cell activation and the resolution of immune responses. For example, the hematopoietic tyrosine phosphatase Lyp, encoded by *PTPN22*, has recently been identified as a major risk factor for several autoimmune diseases, including type I diabetes, autoimmune thyroiditis, systemic lupus erythematosus, myasthenia gravis and RA, with odds ratios in the range of 1.5–2.0 (2, 3). Although expressed in many cell types within the hematopoietic compartment, the best understood function of this phosphatase is to switch off TCR signaling (14-16). Intriguingly, the disease-associated allelic variant, R620W, has been reported to be a gain-of-function mutant paradoxically impairing the association between Lyp and C-terminal Src kinase (CSK), a negative regulator of TCR signals, while at the same time enhancing intrinsic phosphatase activity (17); the sum effect is to increase thresholds of TCR signaling. Whether this impacts on thymic selection or the propagation and/or function of regulatory T cells (Tregs) remains to be determined. However, there does appear to be an association between carriers of the *PTPN22* minor allele and autoantibody production (18, 19).

Finally, there exist associations between RA and polymorphism in genes encoding proteins that serve to regulate immune responses. One of these associations, albeit weak, is with polymorphism at the *CTLA-4* locus, another general susceptibility locus for autoimmunity (3, 13). Surface cytotoxic T-lymphocyte-associated antigen (CTLA)-4 functions as a negative regulator of T-cell activation, binding to CD80/CD86 on APCs, although it can promote the function of Tregs, both features making it a good candidate gene. However, studies suggest that the human risk haplotype may be associated with lower levels of a splice variant encoding a soluble form of CTLA-4, which would serve to block the interaction between the activating co-stimulatory molecule CD28 and its ligands CD80 and CD86 (20). The recently published genome-wide association study suggests that there may be additional RA candidate susceptibility genes likely to influence T-cell function and immune homeostasis, with allelic variation in the regions of the interleukin (IL)-2 receptor alpha (*IL2RA*) and beta (*IL2RB*) chains, as well as the protein tyrosine phosphatase, non-receptor type 2 (*PTPN2*) (3). The fact that these associations have also been documented in other autoimmune diseases, such as type I diabetes, makes them all the more intriguing (3). Together, these data provide compelling evidence to support a role for perturbations in T-cell function in the initiation of RA, and identify potential targets for therapeutic intervention.

Pathways of T-cell activation and differentiation in RA

Over recent years, it has become clear that autoimmune inflammatory arthritis cannot be explained simply in terms of a classical antigen-driven expansion of effector T-cell clones that target synovial joints. Furthermore, pathways of differentiation do not appear to conform to the traditional polarized pathways of T-cell differentiation, as early studies of rodent arthritis models

have implied, although T helper (Th)1 cells expressing interferon (IFN)-γ and tumor necrosis factor (TNF)-α are detected in RA synovial joints in established disease (21, 22). This anomaly may have as much to do with the inflammatory, hypoxic environment in the inflamed synovium, which is known to impair TCR responsiveness (23), as has the accelerated immune senescence that may accompany the 'pre-arthritic' phase of disease (24). Moreover, the precise anatomical site of the early phase of T-cell differentiation may also influence this pathway. A recent analysis of cytokine profiles determined in synovial fluid from patients with very early inflammatory synovitis documented an unexpected Th2 profile, characterized by expression of IL-4, IL-5 and IL-13, while expression profiles in synovial fluid from those patients who subsequently fulfilled the classification criteria for RA lacked these cytokines at detectable levels (25). In established disease, it is generally accepted that synovial T cells express low amounts of IFN-γ and IL-10, as well as TNF-α, while expression of IL-2 and IL-4 is virtually absent (26). The recent finding of an association between RA severity and an IL-4R allelic variant that impairs IL-4R signaling and Th2 differentiation is of particular interest in this regard (27).

New data from animal models of autoimmunity have placed increasing importance on IL-17 expressing $CD4^+$ T cells, known as Th17 cells, and how they might contribute to disease pathogenesis (28). Thus, collagen-induced arthritis is markedly attenuated in IL-17 deficient mice (29), and spontaneous arthritis in IL-1Ra deficient mice can be completely prevented in the absence of IL-17 (30). More recently, the spontaneous arthritis developing in Balb/c mice carrying a point mutation in ZAP-70 has been shown to be completely dependent on the expansion of differentiating Th17 T cells (31). These observations may go some way to explain the disease-exacerbating effects of IFN-γR deficiency in arthritis models on the one hand (32, 33), and the protective effects of IL-6 deficiency or inhibition on the other hand (34), given the reciprocal roles of these cytokines in Th17 differentiation in mice. IL-17 is produced spontaneously in RA synovial cell cultures, and by immunohistochemistry identified in perivascular T-cell rich zones (35). IL-23, which may promote the survival and expansion of Th17 cells, is also detectable in RA synovial joints. The IL-17 receptor is ubiquitously expressed and so may be expected to have pleiotropic effects. Thus, T-cell derived IL-17 (likely to include IL-17A and IL-17F family members) promotes monocyte-dependent IL-1 and TNF-α production (36), induces expression of the osteoclast differentiating factor RANKL (37), and stimulates synovial fibroblasts to express IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), prostaglandin E2 (PGE_2) and matrix metalloproteinases (MMPs) (38). Further studies in RA patients are required to confirm whether T cells expressing IL-17 are crucial effectors of the chronic, persistent phase of synovial inflammatory responses.

Pathways of T-cell effector function

One of the striking characteristics of patients with moderate to severe RA is the presence in the synovial membrane of inflammatory infiltrates that resemble tertiary lymphoid structures, including follicular or germinal center-like reactions (39). These structures likely play a key role in T–B cell cooperation and the local generation of specific autoantibodies. While this pattern of lymphocytic infiltration is found in a subset of patients, it provides robust evidence for ongoing immune reactions at the site of joint inflammation. It is likely that these structures harbor the core cell-to-cell interactions between T cells and B cells essential for immunoglobulin synthesis, as well as those between T cells and macrophages and resident stromal fibroblasts.

There are also a growing number of reports pointing to the importance of T-cell contact-dependent pathways of inflammatory cytokine production by both monocytes and synovial fibroblasts. For example, synovial T cells promote IL-1, TNF, IL-6 and chemokine expression by macrophages in a cell-contact dependent manner that may involve LFA-1–ICAM-1, CD2–LFA-3, CD40L–CD40 and CD69 engagement (40-42). This effect can be reproduced by stimulating T cells from healthy donors with a cocktail of cytokines including IL-2, IL-6, TNF or IL-15. T cells, through direct cell contact, can also stimulate fibroblasts to produce PGE2, MMPs and IL-6 (43, 44), an environment that, at least in mice, would favor the reciprocal activation and differentiation of IL-17 producing T cells. Finally, data support a model where, through expression of TNF-α, IL-17 and RANKL, chronically activated T cells promote bone resorption by augmenting pathways of osteoclast differentiation through their direct action on myeloid precursors (45, 46). Chronically activated synovial T cells, therefore, are key initiators and orchestrators of inflammatory pathways in RA joints, and as such remain valid cellular targets for therapy.

This discussion, above all, highlights the persistence and survival advantage of effector T-cell clones, among other activated cell types. Why chronic immune responses fail to resolve in the susceptible host is not understood. One possible explanation is that effector T cells persist through the failure of immunoregulatory pathways.

Are pathways of immune regulation abnormal in RA?

The primary mechanism that leads to tolerance to self-antigens is thymic deletion of self-reactive T cells. However, since some self-reactive T cells physiologically escape this process (and autoreactive $CD4^+$ T cells are, therefore, present in the peripheral circulation of healthy individuals where they retain their capacity to initiate autoimmune inflammation), negative selection in the thymus is not enough to prevent sustained activation of self-reactive T cells in the periphery. Thus, regulatory mechanisms in the peripheral immune system are required to protect against both the generation of self-directed immune responses and the consequence thereof – the initiation of autoimmune-mediated pathology (47). One such mechanism of peripheral tolerance involves the

active suppression of T-cell responses by CD4+ T cells with potent regulatory capacity. A major subset of these T cells is the CD4+CD25+ regulatory T cell (Treg) subset (48). Tregs are characterized by low proliferative capacity upon triggering of the TCR with polyclonal or allogeneic stimulation *in vitro*, and by their ability to suppress CD4+ and CD8+ T-cell immune responses via cell-contact dependent mechanisms.

In recent years, several studies have proposed that the function of Tregs is severely impaired in autoimmune rheumatic disease, suggesting that in fact a breakdown of Treg-mediated peripheral tolerance may have occurred (49). This would contribute directly to the development of disease by allowing the initial autoimmune response to evolve into a sustained inflammatory response. However, despite this being an attractive hypothesis, these data have to be interpreted with some caution. First, in the absence of a specific surface marker, the identification of Tregs currently still relies on the detection of T cells expressing the transcription factor forkhead box p3 (Foxp3) that in mice has been shown to be highly specific for Tregs (50). However, recent evidence has clearly established that human effector T cells transiently upregulate Foxp3 without imposing regulatory functions (51). Thus, functional studies of Foxp3-expressing T cells in patients with a disease characterized by immunological activity, such as inflammatory rheumatic disease, may in fact include a mixture of Tregs and variable frequencies of contaminating, recently activated effector T cells. Second, it also has been established that *in vitro* assays conventionally employed to assess the regulatory capacity of Tregs do not necessarily reflect their *in vivo* function. For example, whereas Tregs are anergic in these *in vitro* assays, they proliferate vigorously *in vivo* (52). Future studies are clearly required to determine the role of Tregs in human rheumatic diseases more precisely, to analyze their contribution to initiation, perpetuation and regulation of the autoimmune inflammation and also to define their potential role as a therapeutic option to downregulate ongoing inflammation by means of cellular therapy.

A second CD4+ T-cell population with the potential to counteract T-cell driven inflammation in the periphery is the Th2 cell population. Th2 cells, by means of their signature cytokine IL-4, prevent the generation of both Th1 and Th17 cells, and are able to downmodulate their effector functions (53). As outlined above, Th2 cells and their cytokines are virtually absent in established RA, potentially hinting at a pathogenetically important imbalance of T-cell differentiation associated with disease. In fact, it has been shown that T-cell differentiation is severely altered in patients with early RA (54). Whereas an increased generation of IFN-γ producing Th1 cells from uncommitted CD4+ precursor T cells can be detected in *in vitro* assays at the time of disease onset, Th2-cell differentiation is impaired in the majority of patients already at these very early stages of the disease, suggesting that there may exist a profound defect in Th2-cell differentiation in early RA. The data imply that an alteration in the functional ability of T cells to generate immunomodulatory Th2 effectors with the potential to downregulate ongoing Th1-driven inflammation may reflect a breakdown of peripheral tolerance and, thus, may be critically involved in the evolution of sustained rheumatoid inflammation from the outset of the disease-provoking autoimmune response.

Recent data, however, suggest that impaired Th2-cell differentiation is not only critical for the initiation of sustained rheumatoid inflammation, but also impacts on long-term clinical outcome. When patients from the initial T-cell differentiation studies were followed for up to 5 years by repeatedly assessing their disease activity and progression of joint destruction, it became clear that treatment that was conducted at the discretion of an independent rheumatologist induced a meaningful reduction of clinical disease activity in 92% of the patients in whom Th2-cell differentiation could be induced at their first visit, but failed to do so in 64% of the patients with impaired Th2-cell differentiation at disease onset ($\chi^2 = 8.92$, $p < 0.003$). Even more striking was the observation that bone erosions occurred in 36% of the patients who were able to produce Th2 cells, but in as many as 81% of the patients who were unable to generate Th2 effectors ($\chi^2 = 9.01$, $p < 0.003$) despite aggressive drug treatment (Mueller, Skapenko and Schulze-Koops, unpublished observations). These data demonstrate that reduced Th2-cell generation is associated with persistently aggressive and erosive disease, and may, in fact, suggest that a lack of regulatory immune mechanisms, such as the absence of IL-4 producing Th2 cells, contribute to sustained inflammatory activity, which eventually results in severe tissue pathology.

Has T-cell targeted therapy provided evidence for a role of T cells in disease pathogenesis?

If the major role of T cells in RA suggested from the discussion above is of relevance to established clinical disease, we would predict that T-cell directed therapies should be effective. Is this indeed the case? After all, most successful approaches to RA therapy target the effector phase of the disease – either at or downstream of – the monocyte/macrophage level (metho-trexate and TNF blockade being good examples).

While the recent introduction of co-stimulatory blockade with abatacept has re-established the modulation of T-cell co-stimulation as a valid therapeutic approach in RA, this is a particularly relevant question because anti-T cell therapy has not been one of the major success stories of rheumatology until now. Several different antibodies against CD4 have been investigated in the treatment of RA. Direct comparison of the results has been difficult as study design and definitions of clinical response varied greatly. What is evident is that depleting CD4+ T cells with antibodies to CD4 does not result in the sustained reduction of systemic disease activity, or in sustained clinical efficacy. For example, keliximab, a chimeric cynomolgus monkey/human chimeric antibody that depletes CD4+ T lymphocytes, has been evaluated in clinical trials. American College of Rheumatology (ACR) 20 response rates of 47% and 69% were achieved only at the highest dosages, with placebo

response rates of 30% and 19%, respectively (55). Interestingly, the coating rather than the depletion of CD4+ T cells with the antibody to CD4 was found to correlate best with therapeutic responses (55). This prompted attempts to switch from the use of a depleting immunoglobulin (Ig)G1 therapeutic monoclonal antibody (mAb) to the less-depleting IgG4 antibody isotype. One such reagent, clenoliximab was shown to be non-depleting, but to strip CD4 off the surface of T lymphocytes (56). Theoretically, these data suggest modulation of T-lymphocyte function rather than T-cell depletion as the major mode of action. Efficacy results of clenoliximab, however, have not been reported.

A different mAb from CD4, OKTcdr4a, was derived from the murine mAb to CD4, OKT4a, by engrafting its CDR regions onto a human IgG4/κ immunoglobulin (57). A multicenter, placebo-controlled, randomized, double-blind study was initiated in patients with RA refractory to standard therapy with disease-modifying antirheumatic drugs (DMARDs) (58). Clinical response, as assessed by modified Paulus criteria, was achieved after the first treatment week in 67% of the patients who received the anti-CD4 mAb, compared with 25% of the placebo-treated group. Six weeks after treatment, the clinical effect had waned. However, 1 week after the second treatment cycle (*e.g.* after 6 weeks), all patients who had received the mAb had a clinical response, compared with 25% of the patients in the placebo group. There was a significant decrease in C-reactive protein (CRP) levels in all patients 1 week after mAb administration. By contrast, no significant changes were observed after placebo treatment. Remarkably, the administration of OKTcdr4a was not associated with a drop in the numbers of total white blood cells, lymphocytes, neutrophils, monocytes or CD4+ T cells.

Together, the results of these studies support the notion that coating rather than depletion of CD4+ T cells might be effective in ameliorating immunological activity in RA. It is tempting to speculate that the failure of antibodies that deplete CD4+ T cells to show clinical efficacy might relate to the depletion of regulatory CD4+ T cells. Of course an alternative approach to blocking T-cell activation is to prevent the interaction between T cells and accessory cells at the outset of TCR engagement. Such a strategy, which would include co-stimulatory blockade, will be discussed in more detail in subsequent contributions to this review series.

In the context of T-cell targeted therapy, consideration must also be given to conventional DMARDs that have long been believed to act through their effects on T-cell function. Cyclosporin A, a calcineurin inhibitor, mainly targets the Ca^{2+}/nuclear factor of the activated T cell (NFAT) pathway, an essential component for activating the IL-2 promoter. Full activation and survival of activated T cells are, therefore, attenuated by cyclosporin A therapy, which most likely constitutes a therapeutic approach targeting T lymphocytes.

For RA, there is sufficient evidence to suggest that cyclosporin A is effective. In fact, many of the concerns about the drug have had more to do with its toxicity profile rather than its lack of efficacy. In clinical studies, cyclosporine A was superior to placebo at 10 mg/kg, 5 mg/kg and as low as 2.5 mg/kg (59-61). Cyclosporin A also inhibited radiographic progression (60). Patients with inadequate responses to methotrexate benefited from additional cyclosporin A compared with prolonging methotrexate monotherapy (62). In early RA, cyclosporin A in combination with low-dose methotrexate (7.5 mg/week) demonstrated improved ACR20 responses compared with methotrexate (63). Finally, the combination of cyclosporin A and methotrexate was also superior to methotrexate alone with regard to radiographic progression (64). Although cyclosporine A is likely to modify Ca^{2+}/NFAT signaling responses in cell subsets besides T cells, these data have provided a cogent argument for a role of T cells in RA. They do, however, lend further support to the idea that combination therapies that target both lymphocyte and macrophage function may be more effective.

The other DMARD that acts by specifically targeting T cells is leflunomide. By inhibiting the enzyme dihydro-orotate dehydrogenase, the active metabolite of the drug blocks *de novo* pyrimidine synthesis, which is most prominently involved in the proliferation of activated T lymphocytes. While leflunomide may also have effects on other cells, and monocytes/macrophages in particular (65), its major effect is T-cell related. In this regard, it is of interest that leflunomide preferentially inhibits the activation of pro-inflammatory Th1 cells while promoting the differentiation of potentially anti-inflammatory Th2 cells (66).

Leflunomide has been shown to be superior to placebo in large, randomized clinical trials, and as effective as methotrexate or sulfasalazine both in controlling inflammatory disease activity and in retarding radiographic progression (67-69). When added to methotrexate, leflunomide shows additional benefit compared with placebo (70), but it is not clear whether the addition of leflunomide is indeed more efficacious than switching to leflunomide would be (71).

Several trials have proven leflunomide and cyclosporin A to be effective DMARDs for RA, including efficacy with regard to reducing joint erosions. Their mechanisms of action are likely to be distinct; indeed the combination of the two proved to be more effective than either one alone (72). While CD4-directed therapy has not led to a clinical breakthrough to the same extent as the more recent biologic agents, there appears to be evidence of therapeutic efficacy. For none of these three approaches is it presently clear how they might affect the function and/or number of Tregs. Thus, their apparent efficacy could either be explained by differential modulation of effector versus Treg responses, or by a suppression of Treg function in RA, which, as a consequence of the pronounced inflammatory activity, would render Tregs ineffective and attribute lesser importance to their downmodulation in active disease.

This brief review has sought to crystallize a range of issues regarding T-cell activation in RA; these are summarized in the ***Key points box***. It also serves to highlight the need to better understand

the phenomenon of immune homeostasis *in vivo* both at the molecular and cellular levels, and to devise assays to evaluate the dynamics of immune function in patients in response to therapeutic intervention. If this can be achieved in the near future, we should be better placed to examine how new generation T-cell targeted therapies, such as co-stimulatory blockade, exert their beneficial therapeutic effects in the clinic.

Key points box

- The immunogenetics of RA point to a key role for aberrant pathways of T-cell activation in the initiation and/or perpetuation of disease. Besides *HLA-DRB1* and *PTPN22*, susceptibility to disease may be associated with *CTLA-4, IL-2RA, IL-2RB, STAT4, PTPN2* and *PADI4*
- Patterns of lymphoid infiltrates, especially follicular structures with or without germinal centers, provide strong evidence of ongoing immune reactions in established RA
- Spontaneous arthritis in rodents (*e.g.*, K/BxN, IL-1Ra$^{-/-}$, gp130 mutant and SKG mice) have been shown to be T-cell dependent models of disease
- Activation and differentiation of T-helper (Th) cells in RA over time may lead to the accumulation of Th1 as well as IL-17 producing T cells, in association with the impaired differentiation of Th2 cells (at least in established disease)
- More robust markers for CD4$^+$ CD25^{hi} regulatory T cells (Tregs) are required before it can be conclusively established whether or not Tregs are, in relative terms, impaired in number and/or function in RA
- Therapeutic modulation of T-cell function, as opposed to profound immunosuppression or immunodepletion, has been associated with better disease outcomes in clinical trials. Co-stimulatory blockade provides one such strategy for modifying T-cell function
- Defining the distinct effects of co-stimulatory blockade with abatacept on T-cell subsets should provide key insights into understanding how to restore immune homeostasis in patients with RA

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T Cells in Psoriatic Arthritis

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Psoriatic arthritis is characterized by chronic inflammation of the skin and synovial joint. T cells are abundant in the inflamed joint and skin. Disease susceptibility is associated with major histocompatibility complex, which presents antigens to T cells. T cells in the synovial joints have an activated phenotype and demonstrate selective T-cell receptor usage suggestive of oligoclonal expansions. Taken together, these facts suggest that psoriatic arthritis is driven by antigen or autoantigen-driven T-cell activation. The therapeutic benefit of anti–T-cell agents further supports an important pathogenic role for T cells in persistent synovial inflammation and joint damage in psoriatic arthritis.

Introduction

Psoriasis and psoriatic arthritis (PsA) are common. The prevalence of psoriasis is 2% to 4% of the population, and 30% to 40% of psoriatic patients suffer from inflammatory arthritis. PsA is a fascinating rheumatic disease. The occurrence of inflammatory arthritis in patients with psoriasis implies a strong pathogenic link between skin disease and arthritis. However, correlation between joint and skin inflammation is moderate at best. Patients with severe arthritis may have minimal cutaneous disease; conversely, patients with severe skin disease may not suffer from arthritis. In most cases, psoriasis predates the onset of arthritis, but in some patients, arthritis occurs before skin disease. Furthermore, PsA has a different pattern of articular involvement, leading Wright and Moll [1] to categorize it into five subtypes. The ClASsification of Psoriatic ARthritis (CASPAR) group recently revised the classification criteria [2]. The disparity between skin and joint diseases and the heterogeneous pattern of joint involvement suggest a complex interaction among genes, immune response, and environmental factors. Disease prognosis is highly variable, but recent long-term cohort studies have suggested that function disability [3] and joint damage [4] are worse than what has been suggested in standard textbooks.

Evidence Supporting the Importance of T Cells in Psoriasis Pathogenesis

Genetic association

Genetic factors play a major role in patients' susceptibility to psoriasis, especially in those whose disease develops before age 40. Relatives of patients with psoriasis have a risk four to 10 times higher than that of the general population [5]. Psoriasis has an estimated heritability between 60% and 90%. In patients whose psoriasis developed before the age of 40, a stronger association exists with major histocompatibility complex class I molecules HLA-Cw6, HLA-B13, and HLA-Bw57, and class II molecule HLA-DR7. Because the only known function of major histocompatibility complex molecule is to present antigenic peptides to T cells, T cells are strongly implicated in the pathogenesis of psoriasis.

T cells are found in the psoriatic lesions

Psoriasis is characterized by hyperproliferation of keratinocytes and lymphocytic inflammatory infiltrate in the skin [6]. The abundance of activated T cells in psoriatic skin plaques strongly suggests that they are pivotal in disease pathogenesis. The high expression of intercellular adhesion molecule (ICAM)-1 and E-selectin on the endothelial cells in skin lesions facilitates T-lymphocyte trafficking from blood to the psoriatic plaques [7]. Cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, which are found in psoriatic plaque, increase the expression of these adhesion molecules by endothelial cells [8]. One unique feature of T lymphocytes in the psoriatic lesion is the expression of cutaneous lymphocyte antigen (CLA), which binds to E-selectin on endothelial cells [9]. CLA is found in only 10% of circulating T lymphocytes, suggesting that CLA^+ T cells migrate selectively to inflammatory psoriatic skin plaques. Pitzalis et al. [10] examined paired skin and synovial membrane samples from patients with PsA. CLA^+ T cells were found in the skin but not in the joint. Interestingly, the mechanism for accumulating CLA^+ T cells appears independent of E-selectin expression, which was similar in the skin and synovial membrane in patients with PsA.

In psoriasis plaques, T cells have a distinct distribution pattern in which $CD4^+$ lymphocytes are localized to the dermis and $CD8^+$ T cells predominate in the epidermis. Also present in the psoriatic skin lesions are efficient antigen-presenting cells, such as Langerhans and dendritic cells, which are capable of stimulating and activating T cells

[11]. $CD4^+$ T cells have an activated phenotype with expression of CD40 ligand and CD28. Activated T cells release interferon (IFN)-γ, a potent stimulator of macrophages. In turn, IFN-γ releases proinflammatory cytokines (eg, IL-1 and TNF-α), which activate endothelial cells, resulting in further recruitment of T cells into psoriatic plaques.

Anti–T-cell therapy is effective in treating psoriasis

The success of anti–T-cell treatments illustrates the importance of T cells in psoriasis. Alefacept and efalizumab are two anti–T-cell biologic agents licensed for the treatment of psoriasis. Alefacept is a chimeric fusion protein of leukocyte function–associated antigen (LFA)-3 conjugated to the constant region of immunoglobulin G1 that binds to CD2, which is expressed by T cells, especially memory T cells. It deletes $CD2^+$ T cells by inducing apoptosis and natural killer cell–mediated cytolysis [12]. It is administered weekly as a 15-mg intramuscular injection. In a randomized controlled trial, 70% of patients achieved at least 50% improvement in psoriasis area severity index (PASI) after 12 weeks of treatment [13]. Alefacept reduces the number of memory $CD4^+$ and $CD8^+$ T cells in circulation and psoriatic skin lesions, a measure that parallels clinical improvement [14].

Efalizumab is a humanized monoclonal antibody that targets the CD11a subunit of LFA-1, which is expressed by T cells. LFA-1 binds to ICAM-1 and is involved in leukocyte trafficking, T-cell activation, and adherence to connective tissue matrix. Consequently, efalizumab inhibits T-cell activation by blocking costimulation by second signal. It also reduces T-cell trafficking into the diseased skin by preventing the firm adhesion of the leukocyte to endothelial cells [15]. In a double-blind, placebo-controlled trial, 48% of patients treated with efalizumab had greater than 50% reduction in skin disease compared with 15% in the placebo group [16]. An immunohistologic study showed decreased T cells and reduced expression of ICAM-1 in psoriatic plaques.

Evidence Supporting the Importance of T Cells in PsA Pathogenesis

Genetic association

A significant association exists between susceptibility to PsA and HLA alleles: HLA-B27, HLA-B38, HLA-B39, and HLA-Cw6 are increased among people with PsA. HLA-Cw6 is strongly linked with PsA. HLA-B27 is predominantly associated with spondylitis, whereas the others occur more frequently among patients with peripheral arthritis.

T cells are present in the PsA joint

Several studies have compared the immunohistology of the synovia in patients with rheumatoid arthritis (RA) and PsA [17,18]. Van Kuijk et al. [17] took multiple synovial biopsies from patients with PsA and compared them with those taken from patients with RA. T-cell infiltrate was found in both PsA and RA synovia, although T-cell numbers were lower in the former, but the ratio of CD4 to CD8 was similar. Synoviocyte and macrophage numbers were comparable in both diseases. The expression of monokines TNF-α, IL-1, IL-6, and IL-18, as well as that of matrix metalloproteinases, adhesion molecules, and vascular markers were similar for PsA and RA. Recently, lymphoid aggregates akin to germinal centers—which have been well described in RA—were also found on immunohistology of PsA patients [19]. These lymphoid aggregates are composed of T and B cells with coexpression of the chemokines CXCL13 and CCL21. Although the presence of these aggregates was unrelated to disease duration or severity, disease remission was associated with absence of lymphoid aggregates. Costello et al. [20] compared lymphocyte subsets in paired synovial fluid and peripheral blood samples from patients with PsA and RA. They found that the ratio of CD4 to CD8 T cells in PsA is lower than that in RA, especially in the synovial fluid and at the enthesis [21]. The dominance of $CD8^+$ T cells in PsA synovial fluid suggests that they—rather than $CD4^+$ lymphocytes—may be driving the immune response in the joint. This possibility is supported by an association of PsA with HLA class I, as reviewed above.

T-cell function and phenotype in PsA

An interesting study in an animal model by Zenz et al. [22] showed that deletion of *JunB*, a gene localized in the psoriasis susceptibility region PSORS6, led to a phenotype resembling psoriasis with associated arthritis. Interestingly, in contrast to the skin phenotype, the development of arthritic lesions requires T and B cells and signaling through TNF receptor 1 (TNFR1), which implies different pathogenic processes in the development of skin disease and arthritis.

Peripheral blood T cells in patients with PsA are activated

Daoussis et al. [23] examined the expression of CD40 ligand (CD40L), a costimulatory molecule and an early activation marker of T cells in patients with PsA, RA, and normal healthy controls. CD40L expression was increased in stimulated peripheral blood T cells from patients with PsA, especially in those with active disease, compared with RA and normal controls. Cyclosporin suppressed the expression of CD40L expression. CD40L is one of the strongest inducers of T helper (Th) 1 responses, although it stimulates both innate and adaptive immunity. The molecule is normally expressed by activated immune cells such as Th cells that act on dendritic cells to induce their maturation and capability of activating T cells.

Raffeiner et al. [24] found increased expression of Toll-like receptors (TLRs), especially TLR-4 and to a lesser extent TLR-2, on $CD4^+CD28^-$ T cells in patients with PsA when compared with controls [24]. TLRs are important in the innate immune response. They are expressed by immunocytes (ie, lymphocytes, monocytes, neutrophils, dendritic cells, natural killer cells, and B cells). They bind

to bacterial lipopolysaccharides. Receptor engagement leads to the translocation of nuclear factor–κB and to gene transcription of proinflammatory cytokines. In the study by Raffeiner et al. [24], TLR-4 expression was increased in PsA, ankylosing spondylitis, and RA compared with controls. Increased TLR-4 is associated with increased release of perforin, which is cytolytic. TNF-α upregulates expression of TLR-4 and TLR-2. Conversely, TNF-α blockade reduces the expression of TLR-4 and TLR-2. TLR suppression may be one mode of action of TNF-α antagonists in PsA. Also, it may explain the well-known clinical phenomenon in which psoriasis can be precipitated by a streptococcal infection in guttate psoriasis.

Evidence of oligoclonal expansion of synovial T cells in patients with PsA

A small study compared T-cell receptor (TCR) Vβ usage between peripheral blood, skin, and synovia in patients with PsA and found oligoclonal expansion of T cells in the joint [25]. Researchers also found expansion in the skin, although no ubiquitous CDR3 nucleotide sequences were found, suggesting a limited set of conventional antigens may be driving T-cell proliferations.

In a larger study, Costello et al. [26] examined the $CD8^+$ αβ TCR repertoire in paired synovial fluid and peripheral blood of patients with active PsA. Similar to the previous study, this group found oligoclonal expansions in the TCR-β chain, some of which were shared between simultaneous samples of synovial fluid and peripheral blood. Because more expanded clones were found in the synovial joint than in the peripheral blood, these clones were probably generated in the joint. CD4 T-cell oligoclonal expansion was present but in fewer numbers than CD8 T cells, which implies that CD4 T cells participate, perhaps by interacting cognitively to generate the CD8 clones. The same research group extended their work and found that 76% of T-cell clones from PsA joints were polyclonal and unexpanded [27]. Methotrexate decreased T-cell clones that were not structurally related. Hence, it is unlikely that their expansion is antigen driven. Only 12% of the clones were structurally homologous, suggesting antigen drive expansion. These clones were exclusively CD8 in lineage, persisted during methotrexate administration, and were present in both synovial fluid and peripheral blood, which implies that their expansion was antigen driven and that they recognized a yet unidentified PsA antigen or autoantigen.

These observations support the concept of a PsA pathogenesis model in which synovial inflammation is driven by antigens or autoantigens stimulating effector $CD8^+$ and regulatory $CD4^+$ T-cell clonal expansions. However, some researchers have argued that these clonally expanded T cells may not be responding to PsA antigens. They point to evidence suggesting that $CD8^+$ oligoclonal-expanded T cells in the joints are specific for a single epitope from an Epstein-Barr virus lytic cycle protein [28]. Therefore, these activated, virus-specific $CD8^+$ T cells could interact with synoviocytes—either by cell–cell contact or by a cytokine network—and play a bystander role in the maintenance of inflammation in patients with arthritis.

Synovial T cells in PsA are functionally active

A study by Partsch et al. [29] examined the synovial fluid of PsA patients, RA patients, and osteoarthritis controls and found that synovial T cells in PsA are functionally active. Th1 and Th2 T-cell cytokines, IFN, IL-4, TNF-α, and IL-10 were found in PsA compared with osteoarthritis but with less frequency and amount than in RA. This finding was supported by the results of a study by Szodoray et al. [30], in which the levels of 23 circulating cytokines in patients with PsA and healthy individuals were measured using a novel protein array system. They found that serum levels of IL-10, IL-13, IFN-α, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, CCL4 (MIP-1β), and CCL11 (eotaxin) are raised in patients with PsA relative to unaffected controls. These increased cytokine levels suggest broad activation of lymphocytes and monocytes in patients with PsA.

Activated T cells express Receptor Activator of Nuclear factor κB Ligand (RANKL), which provides a key activating stimulus to osteoclasts, resulting in osteoclast differentiation and activation [31]. RANKL binds to RANK expressed by osteoclast precursors and osteoclasts. In the presence of macrophage colony–stimulating factor, RANKL/RANK interaction stimulates osteoclastogenesis, resulting in bone resorption. Osteoprotegerin is a decoy receptor that competes with RANK for binding to RANKL, thereby acting as a natural-occurring antagonist. In PsA synovia, RANKL is found in the synovial lining layer [32•]. RANK-positive osteoclasts are found at the pannus–bone junction. In contrast, osteoprotegerin expression is limited. In a parallel study, osteoclast precursors were found to be increased in the peripheral blood of PsA patients but not in that of healthy controls. Furthermore, when PsA patients were treated with anti-TNF agents, the frequency of osteoclast precursors decreased significantly as early as 2 weeks after treatment initiation. This finding is supported by a study in which freshly isolated peripheral blood and synovial fluid mononuclear cells overexpressed RANKL and TNF-α [33].

The identification of a specific linkage of Th17 in animal models of inflammatory diseases has generated enormous interest and has led to speculation about the role of these cells in the pathogenesis of many human diseases. This topic is discussed in detail by Fitch et al. in this issue.

Anti–T-cell therapy in PsA

Immunohistologic study of the PsA synovia before and after treatment with infliximab showed reduction in T cells in both the skin and synovia [34]. Similarly, the number of macrophages in the synovial sublining layer was significantly reduced after infliximab treatment, although these changes were not due to apoptosis.

Lymphocyte depletion using fludarabine

Fludarabine is a purine analogue licensed for the treatment of chronic lymphocytic leukemia. It inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase. Treatment leads to profound depletion of lymphocytes. In an early phase 2 trial in PsA, 15 patients with active disease refractory to disease-modifying antirheumatic drugs were randomized to fludarabine or placebo. Marked lymphopenia including CD4, CD8, and B cells was observed. American College of Rheumatology (ACR) 20 criteria were met by three of seven fludarabine-treated patients compared with none of the eight placebo-treated patients. Also, three of seven fludarabine-treated patients had more than 20% improvement in the PASI, compared with none of eight placebo-treated patients.

Alefacept in PsA

In an open-label study, alefacept was administered to 11 patients with active PsA [35]. Treatment reduces synovitis and improves symptoms and signs. After treatment, six of 11 patients (55%) fulfilled the disease activity score response criteria. The numbers of lymphocytes (both CD4 and CD8) and CD68 in the synovial tissue were reduced after 12 weeks of treatment.

A subsequent large, randomized, double-blind, placebo-controlled trial studied the efficacy of alefacept in combination with methotrexate in 185 patients with active PsA. Alefacept, 15 mg, or placebo was administered intramuscularly weekly for 12 weeks in combination with methotrexate, followed by 12 weeks of observation during which only methotrexate treatment was continued. At week 24, 54% versus 23% of patients achieved an ACR20 response in the alefacept and placebo groups, respectively ($P < 0.001$). In patients with psoriasis involving at least 3% of body surface area, PASI50 was achieved by 53% in the alefacept group and 17% in those receiving placebo ($P < 0.001$).

Efalizumab in PsA

A recent randomized, double-blind, placebo-controlled trial evaluated the efficacy of efalizumab in 150 patients with active PsA taking concomitant sulfasalazine or methotrexate [36]. After weekly treatment with 1 mg/kg of efalizumab or placebo for 12 weeks, 28% of efalizumab-treated patients achieved ACR20 response compared with 19% of placebo patients. The difference was not statistically significant. The apparent negative effect could be confounded by inadequate dosage, although 1 mg/kg is effective for the treatment of psoriasis, or negative interaction between sulfasalazine and efalizumab.

Conclusions

A substantial body of evidence from genetic association, immunohistologic, and therapeutic studies implicates T cells, especially $CD8^+$ lymphocytes, in the pathogenesis of PsA. The efficacy of anti–T-cell agents in PsA is suggestive but inconclusive; in particular, whether deletion of T cells is important in determining therapeutic benefit will need further research.

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Review article

$CD8^+$ T cells in inflammatory demyelinating disease

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Abstract

We review the contribution made by $CD8^+$ T cells to inflammation in the central nervous system (CNS) in Multiple Sclerosis (MS), and discuss their role in the animal model Experimental Autoimmune Encephalomyelitis (EAE). We show that the inflammatory cytokines interferon-gamma and interleukin-17 are differentially regulated in CNS-infiltrating $CD4^+$ and $CD8^+$ T cells in EAE, and that $CD8^+$ T cells regulate disease. In MS, $CD8^+$ T cells appear to play a role in promotion of disease, so cytokine regulation is likely different in $CD8^+$ T cells in MS and EAE.

Keywords: CD8; Interferon-gamma; Interleukin-17; MS; EAE; T cells

Contents

1. Introduction

Inflammatory T cells are critical to the initiation, progression and regulation of autoimmune pathology in the CNS. The pathology of MS, a progressively debilitating neurological disease of young adults, shows inflammatory demyelination correlated with axonal damage. In support of a role for autoimmune

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inflammation in MS, therapies directed at the inflammatory response have shown benefit (Arnason, 1999). The $CD4^+$ and $CD8^+$ T lymphocytes that are present in demyelinating lesions are proposed to mediate demyelination and axonal damage (Compston and Coles, 2002). This paper will review the evidence that $CD8^+$ T cells play an important role in MS, and describe studies of and discuss the status of $CD8^+$ T cells as a source of inflammatory cytokines in EAE, the most commonly-used animal model for MS.

1.1. $CD8^+$ T cells in MS

Although there is a prevailing opinion that MS is a CD4 T cell-mediated disease, there are data that $CD8^+$ T cells play an important role. As reviewed by Friese and Fugger and by Gold and colleagues, the proportion of $CD8^+$ T cells is higher in MS CNS than in blood, suggesting a selective infiltration process (Friese and Fugger, 2005; Gold et al., 2006). $CD8^+$ T cells are found proximal to damaged and demyelinated axons in CNS, and laser-capture microdissection of such $CD8^+$ T cells allowed TCR CDR3 analysis that suggested oligoclonal expansion of $CD8^+$ T cells in MS lesions, pointing to their active involvement in an immune process (Babbe et al., 2000). Such observations offer a potential resolution to the dilemma that MHC II, which presents antigenic peptides for elicitation of $CD4^+$ T cell effector function, is less widely or prominently expressed on target cells in the CNS than MHC I, which presents peptides to $CD8^+$ T cells (Friese and Fugger, 2005). A role for CD8 T cells, whose functions include cellular cytotoxicity, would help explain mechanisms of myelin and axonal damage in MS.

1.2. $CD8^+$ T cells in EAE

The animal model of MS, EAE, is induced by immunization with antigenic proteins or peptides in adjuvant, or transfer of T cells from mice so immunized. The consensus view is that EAE is a $CD4^+$ T cell-mediated disease (Gold et al., 2006). With rare exceptions the disease can be transferred between animals only with $CD4^+$ T cells, and ablation or blocking studies support that $CD4^+$ T cells are critical for disease. Most studies of the role of $CD8^+$ T cells in EAE describe a regulatory function. Again drawing on the comprehensive review from Friese and Fugger, CD8−/− or CD8-depletion either had no effect or worsened disease, and CD8-deficient mice showed exacerbated disease compared to wild-type animals (Friese and Fugger, 2005; Jiang et al., 1992; Koh et al., 1992). Similarly, EAE experiments in β2-microglobulin−/−mice, in which $CD8^+$ T cells do not develop due to MHC I deficiency, support a regulatory role for these T cells (Linker et al., 2005). The MHC I-like Qa-1 locus, equivalent to HLA-E in humans, restricts peptide recognition by $CD8^+$ T suppressor cells, and these have been implicated in regulation of EAE in mice (Hu et al., 2004). A subpopulation of $CD8^+$ $CD28^-$ regulatory cells has also been described, which can regulate EAE (Najafian et al., 2003).

1.2.1. Induction of EAE by $CD8^+$ T cells

Nevertheless, three labs have reported that EAE can be induced by $CD8^+$ T cells. Immunization of C3H mice with the myelin basic protein (MBP) peptide p79–87 induced a disease that was dominated by infiltrating $CD8^+$ T cells, and which could be transferred by intrathecal injection of $CD8^+$ T cells (Huseby et al., 2001). The MBP epitope was identified using an immunization strategy based on viral expression of MBP so as to elicit responses to 'naturally-processed' MHC I-associated epitopes (Ji and Goverman, 2007). Immunization of C57Bl/6 mice with the myelin oligodendrocyte glycoprotein (MOG) peptide p35–55 was also shown to induce a $CD8^+$ T cell response which could transfer disease to wild-type as well as to T-cell deficient recipient mice (Sun et al., 2001). This same peptide is used to induce $CD4^+$-mediated EAE in C57Bl/6 mice, and has been shown to bind to $I\text{-}A^b$, the MHC II molecule in this mouse strain. So it was significant that a third group showed that a nested peptide MOGp37–46 is a 'pure' D^b (MHC I) epitope, and they could use MHC I tetramers to identify peptide-specific $CD8^+$ T cells in the CNS of mice with EAE (Ford and Evavold, 2005).

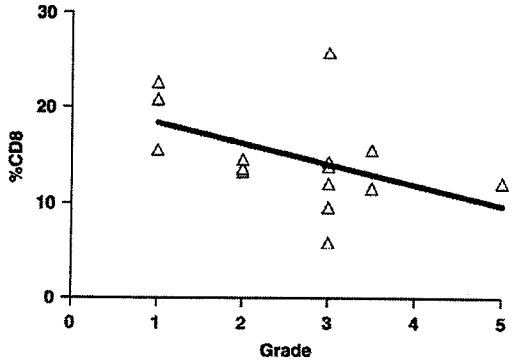

Fig. 1. Mononuclear cells were isolated from the perfused CNS of C57Bl/6 mice with MOGp35–55 induced EAE using methods previously described (Wheeler et al., 2006). Cells were stained with antibodies against CD4 or CD8 as well as anti-CD45 to define $CD45^{high}$ blood-derived infiltrating leukocytes. Relative proportions of $CD4^+$ and $CD8^+$ T cells were measured by flow cytometric analysis (Wheeler et al., 2006). Results are shown as percentages of the total T cell pool that were $CD8^+$, for individual mice, plotted against the severity of disease at the time of collection of CNS-infiltrating cells. The correlation is significant ($p<0.05$, Spearman non-parametric test). Results from three separate experiments are combined in the figure.

1.2.2. Regulation of EAE by $CD8^+$ T cells

We have examined the role of $CD8^+$ T cells in EAE in C57Bl/6 mice immunized with MOGp35–55. Our findings support previous observations in SJL/J mice (Zeine and Owens, 1993), that $CD4^+$ T cells outnumber $CD8^+$ T cells in the CNS of mice with EAE, proportions of $CD8^+$ T cells in the CNS being in the 10–30% range. By contrast in lymph nodes these proportions were equivalent. Interestingly, our data from a number of animals and experiments, show an inverse correlation between proportions of $CD8^+$ T cells and disease severity (Fig. 1). Despite inter-animal variability, this is statistically significant and suggests a regulatory rather than disease-inducing role for $CD8^+$ T cells. A potential mechanism for this could be differential cytokine production by $CD8^+$ versus $CD4^+$ T cells.

1.3. Anti-cytokine therapy in MS and EAE

There was considerable interest in the fact that CD8-induced EAE in C3H mice responded to anti-cytokine therapy more like MS than did CD4-induced EAE. Disease was inhibited by anti-interferon-gamma (IFNγ) antibodies given intrathecally, and was unaffected by a tumor necrosis factor receptor (TNFR)-Fc fusion protein (Huseby et al., 2001). An analogous finding was made in a transgenic mouse in which ectopic expression of the costimulator ligand B7.2/CD86 leads to CD8-dependent CNS inflammation and demyelinating disease. In this mouse, IFNγ-receptor deficiency blocked disease, as opposed to enhancing it, as occurs in CD4-induced EAE (Brisebois et al., 2006). The significance of these observations lies in the very different effects these interventions have had on MS and EAE.

$CD4^+$ T cells that produce the cytokine IFNγ (Th1 cells) are implicated in MS (Compston and Coles, 2002). Administration of IFNγ to relapsing–remitting (RR)–MS patients increased attack rate, which returned to pre-treatment levels when IFNγ was withdrawn (Panitch et al., 1987). This study lacked the magnetic resonance imaging (MRI) oversight which today would be required for such a trial, but the increase in attack rate was indisputable. A more recent study described reduction in disease progression in secondary-progressive (SP)-MS, in patients given antibody against the IFNγ receptor (Skurkovich et al., 2001). Antibody against tumor necrosis factor-alpha (TNFα) was without effect in the same study. However, soluble TNFα receptor therapy had been shown in a separate trial to worsen symptoms and increase MRI lesion incidence in RR–MS patients (1999). TNFα-directed therapy is effective against rheumatoid arthritis (Feldmann and Maini, 2001), and it was striking that some individuals with RA developed neurological disease associated with anti-TNFα therapy (Feldmann and Steinman, 2005; Steinman, 2007; van Oosten et al., 1996). Clearly, this therapy has very different outcomes in MS than in other chronic inflammatory diseases.

1.4. Inflammatory cytokines in EAE

In contrast to MS, administration of IFNγ to rats or mice ameliorated EAE, and anti-IFNγ antibodies made EAE worse. Also mice lacking IFNγ or its receptor showed severe non-remitting EAE (Owens et al., 2001; Willenborg et al, 1996; Steinman, 2007). Despite the observation that transgenic overexpression of IFNγ in the CNS had pro-inflammatory or even frank demyelinating consequence (Owens et al., 2001; Popko et al., 1997), the consensus view is that IFNγ modulates or alleviates EAE. Also in contrast to MS, anti-TNFα ameliorated EAE in mice and TNFRI-deficient mice are relatively resistant to EAE (Owens et al., 2001). Interestingly, the IFNγ and TNFα observations can be linked mechanistically. We showed that IFNγ levels were greatly elevated in the CNS of TNFRI-deficient mice with mild EAE, consistent with a regulatory role for this cytokine and suggesting interplay with TNFα (Wheeler et al., 2006).

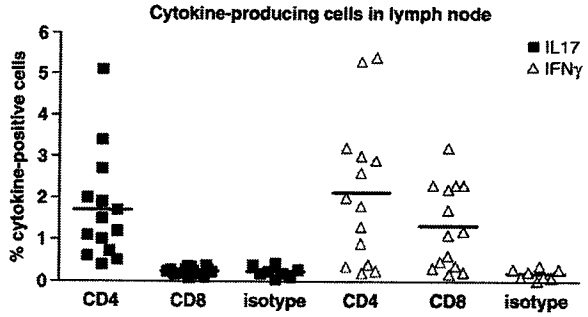

Fig. 2. Leukocytes were isolated from superficial lymph nodes of mice with MOGp35–55-induced EAE (Wheeler et al., 2006). Cells were cultured for 5 h at 10^6 cells/mL in flasks coated with anti-TCRβ antibody, under conditions that promoted intracytoplasmic accumulation of cytokines (as described by Wheeler et al., 2006). Cells were then divided into two pools, permeabilized, and stained with antibodies specific for either IFNγ and IL-17, and then each pool stained for both CD4 or CD8, conjugated to different fluorochromes, as has previously been described (Wheeler et al., 2006). Proportions of $CD4^+$ and $CD8^+$ cytokine-positive cells were determined by flow cytometric analysis, using a FSC/SSC 'live cell' gate and using isotype-matched irrelevant antibodies as a reference gating control. Results for individual mice are shown for each combination of antibody specificities. Results from three separate experiments are combined in the figure. Lymph node $CD4^+$ T cells produced both IFNγ and IL-17 whereas $CD8^+$ T cells only produced IFNγ.

1.5. Interleukin-17 and Th17 cells

The fact that mice lacking one chain of the heterodimeric IFNγ-inducing cytokine IL-12 were resistant to EAE initially suggested a pro-encephalitogenic role for IFNγ. However, it turned out that the p40 chain of IL-12 is shared by another cytokine, IL-23, and it was IL-23 deficiency, not IL-12 deficiency, that explained disease resistance (Cua et al., 2003; Steinman, 2007). Unlike IL-12, IL-23 is not an inducer of IFNγ but instead induces the cytokine IL-17, which has separately been implicated in EAE. It was recently shown that IL-17 deficient mice are relatively resistant to induction of EAE (Komiyama et al., 2006). However, unlike IL-23, IL-17 is not absolutely required for EAE.

Attention has therefore focused to T cells which produce the cytokine IL-17 (McKenzie et al., 2006). In one study, only $CD4^+$ T cells which produced IL-17 (Th17) could transfer EAE (Langrish et al., 2005). It was also shown directly and indirectly that mice lacking the IL-17-inducing cytokine IL-23 were EAE-resistant (Becher et al., 2002; Langrish et al., 2005). In actively-induced EAE, a subset of T cells that produce both IFNγ and IL-17 have been implicated in encephalitogenicity (Suryani and Sutton, 2007). Other studies have also shown that deficiency in IL-17-inducing but not in IFNγ-inducing cytokines prevented EAE (Becher et al., 2002; Chen et al., 2006). The cytokines TGFβ, IL-1 and IL-6 can also promote IL-17 expression, and both IL-1β and IL-6 are critical for induction of EAE (Samoilova et al., 1998; Sutton et al., 2006; Veldhoen et al., 2006). Mice lacking IL-17 develop EAE but onset is delayed and disease is less severe. MOG-induced EAE in IL-17-deficient mice was significantly more dependent on use of pertussis toxin as a co-adjuvant than in wild-type mice (Komiyama et al., 2006). Th17 and Th1 appear to counter-regulate each other's

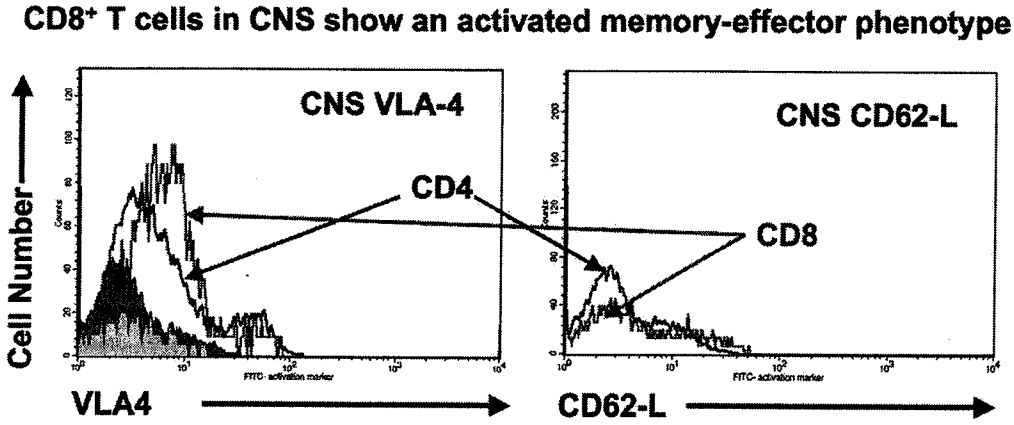

Fig. 3. CNS-infiltrating mononuclear cells were isolated from the perfused CNS of mice with MOGp35–55-induced EAE as described for Fig. 1 and previously (Wheeler et al., 2006). Cells from 3 mice were pooled and stained with antibodies specific for CD4 and CD8, differentiated by different fluorochromes, and also with anti-CD49a (VLA4) or CD62L (L-selectin), and analyzed by flow cytometry. Profiles show numbers of $CD8^+$ (open profile, gray line) and $CD4^+$ (open profile, black line) cells that stained with either of the Mabs specific for activation markers. Filled profiles in the left-hand panel show isotype-matched controls for activation markers on $CD4^+$ T cells (dark gray) and $CD8^+$ T cells (light gray). Isotype-matched controls have been omitted from the right-hand panel since the relevant staining is reduced and therefore would be obscured by them. The figure shows VLA4 staining was increased and L-selectin staining was reduced on both $CD8^+$ and $CD4^+$ CNS-infiltrating T cells. Results were replicated in 2 other experiments (not shown).

production. The proportion of lymph node (LN) $CD8^+$ T cells that produce IL-17 (Tc17) was greatly increased in IFNγ-deficient mice, over a very low number in WT mice (Komiyama et al., 2006). This may help explain why IFNγ-deficient mice have increased susceptibility to EAE (Ferber et al., 1996; Krakowski and Owens, 1996). Also, IL-17-deficient T cells produced more IFNγ (Komiyama et al., 2006). The current view is that IL-17 is pro-encephalitogenic and that IFNγ plays a regulatory role in EAE (Steinman, 2007).

1.6. $CD8^+$ T cells and inflammatory cytokines

$CD8^+$ T cells have long been recognized as a potent source of IFNγ (Kelso and Gough, 1988). Their role as cytokine-producing cells has received less attention than their cytotoxic potential. Nevertheless, the differential response of CD8-induced EAE to IFNγ blockade versus that of CD4-induced EAE led to speculation that the relative predominance of IFNγ-producing $CD8^+$ T cells might account for these differential effects (Steinman, 2001). $CD8^+$ T cells in pooled lymph nodes of mice immunized for EAE did not produce detectable IL-17, unlike $CD4^+$ T cells (Komiyama et al., 2006). Both pools of T cells were strong sources of IFNγ.

1.6.1. Cytokine production by $CD8^+$ T cells in lymph nodes

We have used intracellular cytokine staining to examine whether $CD4^+$ and $CD8^+$ T cells from individual mice with EAE produced IFNγ or IL-17. CNS-infiltrating or LN cells from individual mice were re-activated in vitro using plate-bound anti-TCRβ antibody, stained with antibodies against CD4 and CD8, and then stained (separately) with either anti-IL-17 or anti-IFNγ. In this way we could assess the association of each cytokine with CD4 or CD8. We confirmed by anti-TCRβ staining that CD4 and CD8 were expressed on T cells, and that there was no significant contribution to cytokine staining from non-T cells (such as microglia in the CNS, or macrophages). Proportions of cells staining with anti-cytokine antibodies were evaluated using isotype-matched antibodies as a reference control.

Fig. 2 shows quantification of staining of LN-derived $CD4^+$ and $CD8^+$ T cells with anti-IL-17 and anti-IFNγ, from 14 mice with EAE. Consistent with qPCR findings (see below),

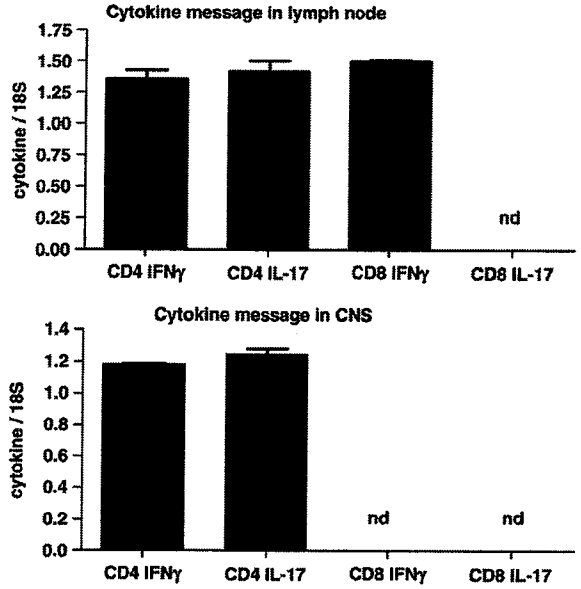

Fig. 4. $CD4^+$ and $CD8^+$ T cells were sorted using flow cytometric cell sorting from the perfused CNS of a pool of 5 mice with MOGp35–55-induced EAE. RNA was isolated from sorted cells and mRNA for 18S rRNA as well as IFNγ and IL-17 was analyzed by qPCR, as described in (Wheeler et al., 2006). RNA levels were normalized to 18S RNA for both pools of cells. Results are shown as mean±SEM of 2 separate experiments. nd=not detected. Message for both cytokines was readily detectable in $CD4^+$ T cells and undetectable in $CD8^+$ T cells.

significant proportions of $CD4^+$ T cells with positive staining for IFNγ and IL-17, as well as $CD8^+$ T cells stained for IFNγ, are readily apparent. By contrast, IL-17 was undetectable in $CD8^+$ T cells, staining of $CD8^+$ T cells with anti-IL-17 antibody being indistinguishable from the isotype control. Variability between animals was considerable, and even within positive groups there were individual mice with very low levels of T cell cytokine. Many of the low-expressors showed low disease severity, but this did not hold for all of them.

1.6.2. Memory-effector status of CNS-infiltrating $CD8^+$ T cells

To address whether $CD8^+$ T cells played an active role in the CNS, as opposed to having bystander status, we determined their activation status by staining for memory-effector markers. We measured expression levels of the adhesion ligands CD49a/VLA4, or α4-integrin, upregulated on activated memory-effector $CD8^+$ T cells, and CD62L/L-selectin, which is down-regulated on tissue-infiltrating inflammatory T cells. Levels of expression of VLA4 and L-selectin were similar on CNS-infiltrating $CD8^+$ and $CD4^+$ T cells, in both cases consistent with an activated, or memory-effector phenotype (Fig. 3). Such a phenotype is usually associated with cytokine production.

1.6.3. Cytokine production by $CD8^+$ T cells in CNS

Real-time RT–PCR (qPCR) analysis of IFNγ and IL-17 mRNA in $CD4^+$ and $CD8^+$ T cells FACS-sorted from the LN and CNS of mice with EAE showed that, whereas $CD4^+$ T cells produced readily detectable levels of both IFNγ and IL-17 message in both LN and CNS, neither message was detectable from $CD8^+$ T cells in the CNS (Fig. 4). However, $CD8^+$ T cells in the LN produced equivalent levels of IFNγ as $CD4^+$ T cells. Limitations on numbers of sorted T cells, especially the minority $CD8^+$ T cells, caution that relative RNA abundance may have contributed to the negative findings for $CD8^+$ T cells in the CNS.

Intracellular cytokine staining of cells isolated from the CNS of individual mice showed that proportions of $CD4^+$ T cells producing IFNγ or IL-17 were higher in the CNS than in LN. The maximum proportions of cytokine-producing $CD4^+$ cells in LN were 5.4% for IFNγ and 5.1% for IL-17 (in the same mouse), mean values being 2.1%±0.5 (SEM, $n=14$) and 1.7%±0.3 (SEM, $n=14$), respectively. In the CNS, maximal proportions of $CD4^+$ cells were 25.0% for IFNγ and 24.6% for IL-17 (in different mice), and the mean values were 15.5%±1.3 (SEM, n=14) and 12.8%±1.6 (SEM, $n=14$), respectively. Percentages of $CD8^+$ T cells producing IFNγ in LN were in the same range as CD4's, the maximum being 3.2% (in the same mouse as showed highest proportions of cytokine-producing $CD4^+$ T cells), with a mean value of 1.3%±0.3 (SEM, $n=14$). There was no correlation between the severity of disease and numbers of cytokine-positive T cells in the CNS.

Because of the low numbers of cells obtainable from CNS of individual mice, percentages of cytokine-positive $CD8^+$ cells in the CNS could be skewed by even quite few non-specific events, and reliable estimates of proportions could not be obtained. It was difficult to discriminate positive events from background in many mice, and IFNγ-producing $CD8^+$ T cells were convincingly stained in the CNS of fewer than 15% of mice. In these mice, about 20% of $CD8^+$ T cells in the CNS produced IFNγ protein.

Taken together with the qPCR analysis, the findings confirm previous reports that $CD8^+$ T cells do not express meaningful levels of IL-17 message or protein, but may be a source of IFNγ. However, $CD8^+$ T cells in the CNS of C57Bl/6 mice with MOGp35–55-induced EAE are at best a poor source of IFNγ, unlike those in LN or the $CD4^+$ T cells in either compartment. Their memory-effector phenotype is consistent with an infiltrating activated T cell subset, as has been shown for $CD4^+$ T cells in the CNS (Zeine and Owens, 1992). This raises questions whether entry of IFNγ-producing $CD8^+$ T cells was selectively inhibited, or more likely, whether the CNS microenvironment does not support reactivation of this cell type or its IFNγ response.

2. Conclusions and discussion

Although it has been reported that $CD8^+$ T cells in peripheral lymphoid tissue do not produce IL-17, ours is the first study to examine CNS-infiltrating CD8's. By analyzing individual mice we could show that some of them contained IFNγ-producing $CD8^+$ T cells. Despite that these were sporadic positives, presumably diluted out in the RNA pools analyzed by qPCR, and the fact that $CD8^+$ T cells are clearly not a major source of IFNγ in the CNS, their detection at all is more consistent with a regulatory role, as was also suggested by the inverse correlation of proportions of $CD8^+$ T cells with disease severity.

These findings, taken together with three reports that $CD8^+$ T cells can be encephalitogenic, raise some questions. Can $CD8^+$ T cells be induced to express IL-17, and did that occur in CD8-induced EAE? These await results of ongoing experiments, but it is likely that under circumstances where CD8 T cells are implicated in induction of CNS inflammation, they would not be an IFNγ-biased cytokine-producing subset. There is now a convincing body of evidence that, in rodents, the cytokine IFNγ exerts a regulatory role, as has been recently reviewed (Steinman, 2007), added to by our recent finding that the regulatory role of IFNγ extends to TNFRI-deficient mice with mild EAE (Wheeler et al., 2006).

This focuses attention to the inflammatory role of IL-17. Studies in rodents have conclusively demonstrated an unambiguous pro-inflammatory role for IL-17 in experimental autoimmune diseases (Steinman, 2007). While not absolutely required for EAE, unlike its inducer cytokine IL-23 (Langrish et al., 2005), IL-17 deficiency rendered mice significantly less susceptible to induction of disease (Komiyama et al., 2006). Requirements for induction of IL-17 include the aforementioned IL-23 as well as TGFβ, IL-1β, and IL-6 (Samoilova et al., 1998; Sutton et al., 2006; Veldhoen et al., 2006). Whether these are also optimal inducers of IL-17 in $CD8^+$ T cells and whether this lineage can be induced to express physiologically significant amounts of IL-17 under any circumstance remains to be determined. Komiyaya et al also showed that some $CD4^+$ T cells produced both IL-17 and IFNγ. This is particularly interesting in light of other data showing that IFNγ appears to counteract induction of IL-17, and that IFNγ mRNA levels were higher in IL-17-deficient mice

(Komiyama et al., 2006), so one awaits information on the role of such double-producer T cells.

The role of IL-17 in human inflammatory disease remains unresolved. In MS, there are now 2 reports of IFNγ administration or blockade, both supporting a pathologic role for this cytokine (Panitch et al., 1987; Skurkovich et al., 2001). It was partly the lack of such evidence in rodents that impelled the research which led to elucidation of the Th17 model. While there are reports showing expression of IL-17 and presence of Th17 cells in human inflammatory disease (reviewed in (Steinman, 2007)), these studies are necessarily of the 'Guilt by Association' type and one must await the results of intervention experiments such as a clinical trial for better causative evidence. For now, it must be acknowledged that the case for a role for IL-17 has largely been made in animal models, not in MS itself. It has been suggested that differential sensitivity of EAE versus MS to IFNγ-based interventions could reflect differential participation of $CD8^+$ T cells, although a mechanism for how this would occur has not been proposed.

Our finding of a negative correlation between $CD8^+$ T cells and disease severity indirectly argues against a significant role for the nested MOGp37–46 peptide that has been reported to drive encephalitogenic $CD8^+$ T cells in mice immunized with MOGp35–55, at least in the context of our immunization protocol. It will be important to determine the extent to which MHC I-binding myelin peptide epitopes are generated, and under what costimulatory circumstances they can induce a $CD8^+$ T cell response. The findings in the present study suggest, that that response would need to include IL-17-producing $CD8^+$ T cells for encephalitogenicity, which by extension implies that such events must have occurred in MS.

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T Cells in the Pathogenesis of Type 1 Diabetes

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T lymphocytes' crucial role in the autoimmune process leading to insulin-dependent type 1 diabetes is now universally recognized. Research focuses on identifying pathogenic and nonpathogenic T cells, understanding how they are primed and expanded, characterizing their antigen specificity, and ultimately on devising strategies to blunt their autoaggressive action. In this review, we focus on recent progress identified in three different areas. Results obtained with transgenic mice acknowledge proinsulin's unique role in triggering autoimmunity and suggest that other β-cell proteins are recognized as a result of epitope spreading, at least in the nonobese diabetic mouse. Progress has also been achieved by developing and validating reliable CD4+ and CD8+ T-cell tests that may prove valuable for diagnostic and prognostic purposes in the near future. Finally, recent results provide novel and important guidance for manipulating autoreactive T-cell responses against β-cell antigens.

Introduction

Type 1 diabetes (T1D) is a T-cell–mediated autoimmune disease targeting the insulin-producing β cells of the pancreas. It has taken time to make this common knowledge.

The emergence of this notion has followed a twisted intellectual journey. First, it was not evident until the 1970s that T1D was an autoimmune disease. The work of Bottazzo and the discovery of islet cell antibodies (ICAs) yielded the concept of an autoimmune pathogenesis [1]. Following the paradigms of other diseases such as myasthenia gravis and Graves' thyroiditis, attention then focused on autoantibodies' pathogenic role. The ensuing studies have been important for advancing research on T1D pathogenesis because essentially all antigens (Ags) targeted by autoreactive T lymphocytes have initially been identified as targets of autoantibodies. However, it is widely accepted today that the anti-islet autoantibodies do not play an essential role, with the possible exception of a modulating effect on the presentation of β-cell Ags [2]. In humans, a case report describing T1D development in a patient affected by X-linked agammaglobulinemia documented the dispensable nature of β-cell autoantibodies, although this does not rule out an adjuvant role of enhanced autoantigen presentation mediated by antibodies in other cases [3].

However, it was the availability of the nonobese diabetic (NOD) mouse model in the 1980s that helped clarify T lymphocytes' central pathogenetic role. Several reports have greatly advanced our understanding of how this T-cell pathogenesis takes place.

CD4+ or CD8+ T Cells?

In light of the strong association between the major histocompatibility complex (MHC) class II locus and T1D, CD4+ T cells have long mesmerized investigators. Frequently, several investigators could isolate from lymphocytic islet infiltrates bulk and cloned T-cell populations causing disease upon transfer into lymphopenic NOD mice or when expressed in transgenic animals. The β-cell Ags recognized by several such clones, including clone BDC2.5, the most widely used and studied diabetogenic CD4+ T cell, remain to be identified [4]. However, data gathered more recently point to a parallel critical role of CD8+ T cells in T1D pathogenesis. NOD mice lacking MHC class I molecules [5–7] or injected with anti-CD8 monoclonal antibodies [8] do not develop insulitis. More importantly, CD8+ T cells specific for insulin $B_{15\text{-}23}$ [9] and for islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206\text{-}214}$ [10] are early and critical actors in the T1D pathogenesis of NOD mice [11,12].

Overall, CD4+ and CD8+ T cells are required for progression to T1D [13–15], and diabetogenic clones (ie, clones capable of accelerating or inducing T1D upon adoptive transfer in NOD or NOD/SCID recipients) have been described for the CD4+ and CD8+ subset. Three different CD8+ T-cell clones derived from (pre)diabetic mice and specific for insulin $B_{15\text{-}23}$ [9], $IGRP_{206\text{-}214}$ [14] and dystrophia myotonica kinase $(DMK)_{138\text{-}146}$ [16] have been reported (also referred to as clones G9C8, 8.3, and

AI4, respectively). For each of these clones, transgenic T-cell lines expressing the relevant receptors have been produced and represent important tools for studying the role of $CD8^+$ T cells in T1D. Interestingly, the insulin B_{15-23} $CD8^+$ T-cell epitope overlaps with a B_{9-23} $CD4^+$ epitope previously described as the target of a highly diabetogenic $CD4^+$ T-cell clone [17].

Is There an Initiating Autoantigen/Epitope?

Proinsulin (PI) is the only T1D autoantigen expressed, next to thymic epithelial cells involved in T-cell education, exclusively in β cells. PI's importance as an early target Ag is supported by data on PI knockout NOD mice. Different from humans, rodents express two PI isoforms, referred to as PI1 and PI2. NOD mice defective for the PI2 gene, the prevalent isoform in the thymus, display accelerated T1D, likely related to defective deletion of PI-reactive T cells [18]. Consistent with this hypothesis, NOD mice are protected from diabetes when a PI transgene is inserted that deletes the specific T cells in the thymus [19]. Conversely, NOD mice defective for the PI1 gene, the prevalent isoform in the islets but lacking expression in the thymus, are less susceptible to T1D. However, PI1 knockout islets transplanted into recently diabetic wild-type NOD mice become infiltrated and only transiently reverse T1D, suggesting that PI is an early but not exclusive target [20].

Recent evidence from Nakayama et al. [21••] further suggests that PI may be the initiating β-cell Ag in T1D. These authors produced NOD mice where the endogenous PI1 and PI2 genes have been deleted and replaced by a hormonally active PI transgene carrying a single amino acid mutation at position B16 (tyrosine to alanine). These mice are completely protected from T1D and insulitis [21••]. Intriguingly, the introduced substitution affects PI recognition by $CD4^+$ and $CD8^+$ T cells: position B16 is an anchor for binding to the H-2 K^d in the immunodominant $CD8^+$ epitope PI_{B15-23} and an invariable T-cell contact in the equally immunodominant $CD4^+$ T-cell epitope PI_{B9-23}. These data suggest that recognizing these immunodominant epitopes by $CD4^+$ and/or $CD8^+$ may be a mandatory early event in T1D pathogenesis.

To understand the mechanism of protection from islet autoimmunity by the B16 substitution, Nakayama et al. [22] undertook a series of adoptive transfer and immunization studies. First, an active tolerizing effect of transgene expression (eg, due to promoter leakiness or to increased thymic PI expression) could be ruled out because transgenic expression of a PI molecule with the native Tyr16 restored insulitis fully. Further experimentation suggested that the critical sequence in the PI B chain acts at two different levels: 1) for the initial priming of NOD anti-islet autoimmunity, as transplantation of NOD islets, but not bone marrow, expressing native PI sequences into mice carrying the PI-B16Ala transgene rapidly restored development of insulin autoantibodies and lymphocytic infiltration of recipients' islets carrying mutant PI sequences; and 2) for the effector phase, as splenocytes from B16-mutated mice transplanted with PI wild-type NOD islets induced T1D when transferred into wild-type NOD/SCID or even into B16-mutated NOD/SCID mice. Also, splenocytes from mice immunized with native insulin B_{9-23} peptide induced rapid T1D upon transfer only in recipients expressing the native PI sequence in their pancreata. Additionally, $CD4^+$ T cells from B16-mutant mice immunized with native insulin B_{9-23} peptide promoted insulin autoantibodies in NOD/SCID mice. Therefore, the provision of the native insulin B chain sequence is sufficient to prime anti-insulin autoimmunity, whereas subsequent transfer of T1D after peptide immunization requires native insulin B chain expression in islets [22].

Studies by Krishnamurthy et al. [23•] further corroborated the hypothesis that PI is the initiating Ag in the T1D of the NOD mouse, because mice rendered tolerant to PI by transgenic overexpression of PI2 in Ag-presenting cells do not develop the immunodominant $IGRP_{206-214}$-specific responses and are protected from T1D. Conversely, mice made tolerant to IGRP by the same means are not protected from T1D [23•], suggesting that the IGRP-specific responses lay downstream of PI-specific ones in the pathogenic cascade. The prerequisite of PI-specific responses for T1D to develop is also found in NOD8.3 mice, which are transgenic for a T-cell receptor recognizing the $IGRP_{206-214}$ epitope.

Epitope Spreading and "Secondary" Autoantigens

Despite strong evidence pointing to PI's triggering role, identifying more autoantigens and examining T-cell responses to them remain a high-priority goal of T1D research. Several considerations justify these efforts. First, the evidence supporting PI's critical role has been obtained in the NOD model, and T1D pathogenesis may be different in humans. Second, once autoimmune T-cell (and B-cell) responses to β cells are initiated, the specificity of these responses rapidly enlarges to include more Ags, a phenomenon referred to as epitope spreading. As a result of this process, responses to the presumable triggering Ag can rapidly be "overgrown" by a secondary response, as exemplified by IGRP-specific $CD8^+$ T cells, which rapidly outnumber PI-specific cells in the NOD model [10]. Finally, in the inbred NOD mouse, some variation exists in the specificity of the $CD8^+$ T-cell response between individual mice, a minority of which displays dominant responses to the DMK Ag [24]. Such variation is more extensive in the outbred human population. Because epitope spreading may occur in all individuals displaying clear signs of β-cell autoimmunity and, therefore, eligible for Ag-specific immune intervention strategies that may become available in the future, numerous autoantigens must be studied to cover individual variation in the specificity of autoimmune responses.

Although several autoantigens recognized by diabetogenic CD4+ T cells remain to be identified [4], recent data allow better assessment of two major autoantigens targeted by β-cell autoimmunity in NOD mice and humans: 65-kDa glutamic acid decarboxylase (GAD65) and tyrosine phosphatase-like molecule 512/insulinoma-associated protein 2 (IA-2). Knockout of the latter Ag in NOD mice did not prevent cyclophosphamide-induced diabetes, suggesting that IA-2, although required for normal glucose-stimulated insulin secretion, is dispensable for T1D development [25]. Using an even more conclusive approach, Jaeckel et al. [26] demonstrated that efficient tolerization of GAD65-specific CD4+ T cells by transgenic GAD expression in professional Ag-presenting cells of NOD mice did not affect T1D incidence or time of onset, suggesting that autoimmunity to this protein emerges downstream of earlier triggering events involving other Ags. Conversely, when the same authors used a similar approach to induce PI tolerance, T1D incidence was reduced. That some mice still developed T1D was interpreted as evidence that autoimmunity to PI is not an absolute requirement for T1D development. However, the fate of PI-specific CD8+ T cells was not addressed, and experimental evidence for tolerization of CD4+ T cells was more difficult to obtain in this study than in the previous one concerning GAD65. Consequently, PI tolerance may not have been complete in PI-transgenic mice [27]. Thus, considering current published evidence, only PI appears to be a plausible candidate for the role of a triggering autoantigen in the NOD mouse.

What Is the Mechanism of β-Cell Destruction?

Han et al. [28••] proposed an avidity maturation model in which the autoimmune evolution toward full-blown T1D requires the gradual selection of β-cell–specific CD8+ clonotypes of higher avidity [10]. Blunting of this avidity maturation process by peptide treatment effectively prevents disease. The corollary to this model is that peptide therapy should target immunodominant epitopes with peptides of limited affinity and/or in limited amounts. As shown for the $IGRP_{206\text{-}214}$ immunodominant epitope of NOD mice, this is important to spare low-avidity clonotypes, which can thus expand to occupy the intra-islet space left free by their high-avidity counterparts [28••]. Otherwise, near complete deletion of the epitope-reactive T-cell pool enhances the recruitment of subdominant (yet pathogenic) specificities, which then can cause disease. This model may explain why previous attempts to silence human autoimmunity by peptide injection were unsuccessful [29] but seemed to work better at lower doses [30]. Peptide therapy in autoimmunity may be most effective under conditions that foster occupation of the target organ lymphocyte niche by nonpathogenic, low-avidity immunodominant clonotypes than by pathogenic subdominant ones. It remains to be seen whether protection from T1D by low-affinity clones is a passive phenomenon, based on simple occupation by such clones of lymphoid "space," or involves an active regulatory mechanism.

The mechanisms responsible for the final damage to the β cells remain uncertain. The fact that CD4+ and CD8+ T-cell clones can mediate T1D development under certain conditions may suggest that cytokine- and cytotoxicity-mediated mechanisms are taking place. Besides a direct toxic effect on β cells, the role of Th1 cytokines may also be to "sensitize" β cells for killing by inducing Fas expression [31,32]. The cytotoxic activity may also be shared by β-cell–specific CD4+ T cells, as CD4+ T cells can also differentiate into cytotoxic T lymphocytes (CTLs) [33]. The lack of class II expression on β cells makes the Fas pathway more likely for CD4+ CTLs after their Ag-specific activation [34]. Perforin- and Fas-mediated killing mechanisms may instead be at play for CD8+ T cells [35,36].

What Is the Evidence in Human T1D?

In contrast to all evidence in the NOD mouse, formal demonstration for T cells' pathogenic role in human T1D is lacking. We know that CD4+ and CD8+ T cells are abundant in the T1D insulitis infiltrates [37] and that detecting CD4+ and CD8+ T-cell responses may offer new autoimmune markers for clinical applications [38,39,40•]. However, assessing whether β-cell–specific T cells are pathogenic would require demonstrating cytotoxic activity in vitro and/or of pathogenic potential in adoptive transfer experiments in humanized mouse models.

Another open issue is whether a similar Ag/epitope hierarchy can be established for human T1D, as observed in the NOD mouse. Is PI also the initiating Ag for human disease? Only indirect evidence suggests that this could be the case. The genomic locus conferring T1D susceptibility ranking second after the HLA class II region is a variable number of tandem repeats (VNTR) upstream of the PI gene promoter. Although the precise mechanism underlying increased T1D susceptibility is unknown, protective (class III) VNTR alleles are associated with higher PI mRNA and protein expression in the thymus and, likely, more efficient deletion of PI-reactive T cells [41]. Even though a recent clinical trial with subcutaneous insulin in at-risk subjects was unsuccessful [42], other regimens formulated from more recent data on Ag-based therapy [28••] may bring different results. Longitudinal analysis of the evolving β-cell–specific T-cell responses throughout the healthy prediabetic and diabetic period in selected high-risk subjects may also help to clarify this point.

T-Cell Assays in Humans

Although direct evidence for a pathogenic or otherwise disease-modifying role of specific T cells will be difficult to obtain in humans, assays allowing for quantitative detec-

tion and phenotypic and functional characterization of T cells recognizing β-cell Ags can provide information with diagnostic, prognostic, and therapeutic relevance in managing patients with T1D or at high risk of developing it. Such assays might help to assess the disease risk in concert with, or independently of, autoantibody measurements. Moreover, they might provide guidance for immunointervention strategies (eg, by helping to choose the time point for intervention and/or the Ag targeted by it). Another possible application of such tests might be monitoring of immune intervention that if successful would be expected to alter the frequency and/or phenotype of autoantigen-specific T cells. Although developing reliable T-cell assays has been a research goal for many years, substantial progress has only been seen in recent years.

Assays measuring CD4+ T-cell responses, long plagued by poor specificity and/or sensitivity, have recently been evaluated in a blinded fashion in a study sponsored by the Immune Tolerance Network. Whereas a standard proliferation assay using soluble Ags and interleukin-2 addition showed excellent specificity (94%) but poor sensitivity (58%), an assay measuring proliferation to human pancreatic proteins added to cultures after blotting on nitrocellulose particles displayed excellent specificity (83%) and sensitivity (91%) [40•]. Thus, an assay using many of the identified autoantigens (PI, GAD, IA-2, and others) showed much lower sensitivity than one using bulk β-cell Ags, which may suggest that Ags remaining to be identified account for a significant proportion of β-cell targeted autoimmunity at T1D onset. Although the source of antigenic material used in the latter assay (islets prepared from cadaveric human pancreata) may not conveniently be obtainable for more widespread use of the assay, it is encouraging that at least one group can distinguish T1D patients and healthy individuals reliably in a CD4+ T-cell assay. It is hoped that additional and less cumbersome assays (eg, ones reported to detect secretion of different cytokines by CD4+ T cells from patients and controls in response to IA-2 and PI peptides [38]) will be validated in a similar fashion in the near future.

Impressive progress has recently been achieved by several groups with CD8+ T-cell responses against β-cell Ags. We initially used an enhanced "reverse immunology strategy" to identify six naturally processed, HLA-A2–presented epitopes derived from PI [43•]. More epitopes derived from GAD65 and IA-2 were identified upon DNA vaccination of HLA-A2 transgenic mice, followed by screening with splenocytes from immunized mice of candidate epitopes selected by a prediction algorithm [44]. Using the resulting panel of 20 epitopes, we found that an Elispot assay measuring interferon-γ secretion by peripheral blood mononuclear cells from HLA-A2+ donors could discriminate patients at T1D onset and controls, with sensitivity (86%) and specificity (91%) similar to that seen with the immunoblot test discussed above [39•]. Although these results remain to be validated with a larger number of patients and in blinded fashion, they suggest that CD4+ and CD8+ T-cell tests capable of reliable and specific detection of T1D-associated autoimmunity may be at hand. Once tests are validated, it will be a priority to analyze T cells from individuals at high risk for T1D and to include pediatric patients in screening. The latter will be facilitated by the fact that the performance of our Elispot assay is identical when a restricted panel of five epitopes is used.

Other groups also identified HLA class I (mainly HLA-A2) presented autoantigenic epitopes recognized by patient CTLs [45–48]. Even though most of these studies did not provide statistically validated information on the suitability of the discovered epitopes for distinguishing patients from controls, covering a wide array of Ags and epitopes will be helpful for developing tests with wide population coverage. An interesting study published very recently found that tetramers of HLA-A2 with peptide $GAD_{114-122}$, an epitope also immunodominant in our study, detect the same number of specific CD8+ T cells in patients and controls; however, in patients (and not controls), many of the specific cells were found in the activated/memory CD45RO+ fraction [49•]. Reconciling this report with our finding of exclusive responses to this and other epitopes by patient CTLs, naïve-specific cells from healthy donors would not be expected to secrete interferon-γ.

Conclusions

Although the notion of T1D as an autoimmune disease first stemmed from the description of ICAs in patients, evidence gathered to date about the T-cell–mediated T1D pathogenetic mechanisms comes from the NOD mouse model. Validating and transferring this knowledge to human T1D will further deepen our understanding of the disease and may provide new inspiration for therapeutic interventions.

Disclosures

No potential conflicts of interest relevant to this article were reported.

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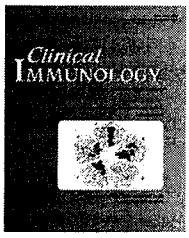

SHORT ANALYTICAL REVIEW

Down-regulation of autoreactive T-cells by HMG CoA reductase inhibitors

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Abstract The inhibitors of HMG CoA reductase (statins) are widely used as cholesterol-lowering drugs with excellent safety records in hypercholesterolemic patients. Statins exert pleiotropic effects on a variety of cells, and they were recently described as a new class of immune modulators. Depending on their structure, dose, and route of administration, statins regulate the function of both the antigen-presenting cells and T-cells by HMG CoA reductase-dependent and independent mechanisms. Herein, we describe these mechanisms leading to prevention, amelioration, and reversal of autoimmune diseases. We also present data from our laboratories showing for the first time that in a double transgenic mouse model for autoimmune diabetes, atorvastatin (lipitor) prevented the onset of disease when administered in the neonatal period, and stabilized the glucose levels when administered in mice developing a mild form of diabetes.

Introduction

The inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (statins) are well known to improve prognosis in patients with high cholesterol and atherosclerotic cardiovascular disease. Clinical trials in this area are numerous and involve a variety of patients with or without overt evidence of vascular problems. Heart attacks, anginal symptoms, and strokes are all reduced. Suggested mechanisms of benefit include removal of lipid from plaque, restoration of nitric oxide synthesis, and anti-inflammatory properties. Statins such as lovastatin, pravastatin, simvastatin, fluvastatin, and atorvastatin are currently used as cholesterol lowering drugs with excellent safety records in patients with hypercholesterolemia [1–3].

The endoplasmic reticulum (ER)-bound HMG CoA reductase is the rate-limiting factor for cholesterol biosynthesis [4] (Fig. 1). Early observations in mice and rats indicated that HMG CoA reductase is mostly expressed in liver. Today, it is known that HMG CoA reductase is expressed in virtually

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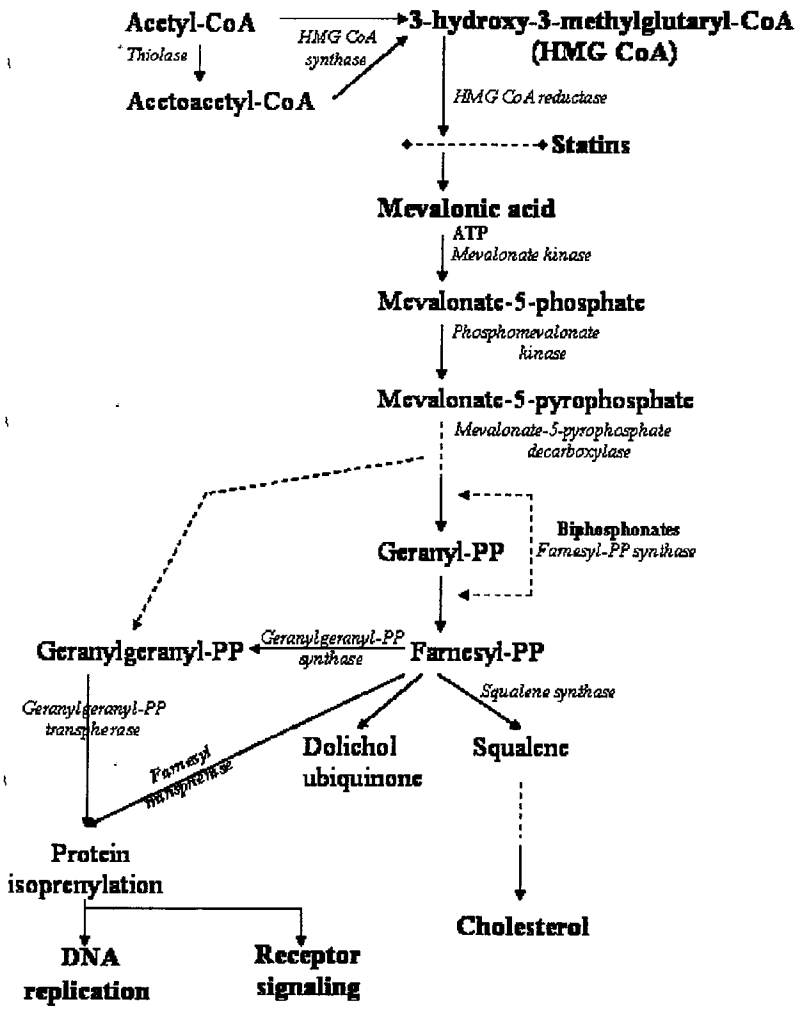

Figure 1 Mevalonate pathway of cholesterol synthesis.

all tissues, including lymphocytes [5]. In fact, the HMG CoA reductase activity was first measured in lymphocytes. The role of this enzyme in various cells (that are not the major source for cholesterol) may well explain the need for intracellular and plasma membrane cholesterol as an important constituent of lipid rafts [6]. The lipid rafts are insoluble microdomains in the plasma membrane that constitutively incorporate, or recruit under various circumstances protein receptors involved in cell signaling mediating cell growth, functional activities, and cell death [7]. The HMG CoA reductase enzyme is an AMP-activated protein kinase whose catalytic activity depends greatly on its phosphorylation/dephosphorylation status [8]. Phosphorylation lowers the HMG CoA reductase catalytic activity and increases the susceptibility to degradation [9]. Mevalonate, which is a major metabolite of HMG CoA reductase activity, is actively involved in the normal feedback regulation of HMG CoA reductase. Mevalonate as well as 25-hydroxycholesterol, low-density lipoproteins, insulin, glucagon, thyroid hormone and estrogens, can regulate HMG CoA reductase activity at transcriptional and translational levels [10].

The primary effect of statins on lowering the blood cholesterol (by as much as 65% in the case of atorvastatin) is mainly attributed to inhibition of HMG CoA reductase [11,12]. Besides their cholesterol-lowering effect, statins exert pleiotropic effects on various types of cells, among them antigen-presenting cells (APC) and T-cells. These effects may or may not depend on the HMG CoA reductase pathway. That is because HMG CoA reductase leads not only to cholesterol synthesis, but also to the generation of several isoprenoid intermediates. Isoprenylation of proteins has an important role in cell signaling. It is mediated by prenyltransferases that covalently link the farnesyl and geranylgeranyl groups (isoprenoids) to the proteins, and enable them to relocate to the plasma membrane. Inhibition of protein prenylation by statins leads to inactivation of small GTP signaling proteins such as Ras. These proteins are signal transducers involved in lymphocyte activation. Ras proteins are farnesylated, whereas Rho, Rac, and Rab proteins are geranyl-geranylated or prenylated. Ras and Rho proteins connect the extracellular stimuli with downstream kinases such as MAPK, ERK, c-Jun N-terminal kinase (JNK), and p38αMAPK [13]. ERK is mostly activated by mitogens, whereas JNK and p38αMAPK are activated by stress-inducing factors and pro-inflammatory cytokines such as INF-γ and TNFα. It has thus been suggested that statins-

mediated inhibition of protein isoprenylation is a major mechanism responsible for suppression of lymphocyte function. Such effect is highly desirable in autoimmune diseases and allograft rejection [14,15].

Modulation of T-cell function by statins

Studies of the effects of statins led to the conclusion that these drugs represent a new class of immune modulators. The interaction between the major histocompatibility complex (MHC) charged with antigenic peptides on antigen presenting cells (APC), and the T cell receptor (TCR) on T-cells (signal 1) is known to mediate T-cell responses in mammalians. At the same time, more than 40 different accessory molecules on APCs interact with their ligands on T-cells to provide adhesion and enhance the T-cell response (signal 2, costimulation). Among these, LFA/ICAM-1 and CD40/CD40L interactions play an important role. However, the archetype of T-cell costimulation is the interaction of CD80 and CD86 (B7.1 and B7.2) on APCs with their CD28 receptor on T-cells.

Statins can alter both signal 1 and 2 of T-cell activation, either indirectly by interfering with APC function, or directly by affecting T-cell function through mechanisms that may or may not involve HMG CoA reductase pathway of cholesterol synthesis.

Effects of statins on APCs

Deficient antigen presentation to T-cells through MHC II molecules on APCs can negatively regulate the T-cell function. It is well know that the MHC class II molecules are constitutively expressed on professional APCs, i.e., dendritic cells and B lymphocytes, but their expression can also be induced by IFN-γ on endothelial cells, myocytes. fibrocytes, and microglia [16]. Kwak et al. studied the regulatory effect of atorvastatin on both MHC II expression on professional APCs and IFN-γ-induced MHC II expression on human endothelial cells and monocytes [15]. The authors found that atorvastatin effectively repressed induction of MHC II expression by IFN-γ without affecting the constitutive expression of MHC II on professional APCs. The addition of mevalonate to the cell cultures abolished the inhibitory effect of atorvastatin, suggesting that blockade of HMG CoA reductase is part of these statin-induced negative regulatory mechanisms. Atorvastatin repression of IFN-γ-inducible MHC II expression was attributed to inhibition of the inducible promoter IV of CIITA. The CIITA transactivator is critical for up-regulation of MHC II expression by IFN-γ [17]. It thus appears that inhibition of MHC class II expression on APCs is a mechanism by which statins can lower the amplitude of a T-cell response by minimizing the extent of antigen presentation to T-cells by APCs. Neuhaus et al. found that atorvastatin can also inhibit the constitutive expression of MHC class II molecules on human B cells [18]. Simvastatin was reported to inhibit the IFN-γ-inducible CIITA promoter leading to lower expression of MHC class II in human vascular endothelial cells [19]. The authors further concluded that atorvastatin did not affect MHC I expression.

Another negative regulatory effect of statins on APCs may relate to inhibition of migration and secretion of inflammatory factors. Obviously, lower number of APCs at the site where T-cells accumulate and receive stimulation by APCs and a lack of inflammatory factors in the milieu can impair T-cell activation and proliferation. Macrophages can process and present tissue antigens. Their migration and activation depend on several matrix metalloproteinases such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (gelatinase-B) [20,21]. Fluvastatin was shown to inhibit expression of MMP-9 in vitro [22] and accumulation of macrophages in vitro and in vivo [23] through down-regulation of E-selectin and ICAM-1 expression on endothelial cells [24].

Reduction in T-cell activation by APCs may be also achieved by impairing APC-T cell adhesion, e.g., through reducing interaction of ICAM-1 on epithelial cells with LFA-1 on T-cells. Epithelial cells are known to present antigen to T-cells, and to up-regulate expression of MHC class II molecules upon stimulation with IFN-γ. Moreover, LFA-1 not only promotes adhesion of T-cells to APCs but also provides costimulation to T-cells upon ligation by ICAM-1. Lovastatin and mevastatin can reduce the LFA-1-/ICAM-1 mediated adhesion and its costimulatory function in T-cells [25]. Kallen et al. noted that inhibition of LFA-1/ICAM-1 interaction relies on binding of lovastatin to a conserved domain of LFA-1, namely the CD11a I-domain [26]. Also, cerivastatin, a chemically synthesized statin, was able to suppress expression of ICAM-1 on APCs in Lewis rats and prevent acute graft rejection of allografts from Fisher rats [27].

Deficient costimulation of T-cells provided by APCs is another mechanism by which APC function can lower T-cell activation. The CD40 expression on APCs, and particularly on endothelial cells, is up-regulated by INF-γ and TNFα inflammatory cytokines. Atorvastatin, cerivastatin, and pravastatin were able to reduce by 50% the INF-γ and TNFα-induced CD40 expression on endothelial cells through mechanisms independent of HMG CoA reductase inhibition. This was concluded because addition of mevalonic acid did not reverse the inhibitory effect of these statins on endothelial cell CD40 expression [28]. These mechanisms appeared to rely on inhibition of NF-κB and STAT-1 transcription factors that regulate expression of interferon regulatory factor 1 (IRF-1), which in turn, governs the IFN-γ-inducible expression of CD40. In contrast, the same drug atorvastatin inhibited the IFN-γ-inducible expression of CD40, CD80, and CD86 on microglia by an HMG CoA reductase-dependent mechanism [29].

A low proliferative capacity of APCs will subsequently result in a low number of APCs able to present antigen to T-cells, which, in turn, leads to deficient T-cell activation. The oxidized low-density lipoprotein (Ox-LDL) induces macrophage proliferation through secretion of granulocyte/macrophage colony-stimulating factor (GM-CSF) and subsequent activation of p38αMAPK. Cerivastatin and simvastatin were found to inhibit Ox-LDL-induced proliferation of macrophages but not Ox-LDL-induced GM-CSF production [30]. In fact, these statins inhibited the GM-CSF-dependent p38αMAPK by a mechanism relying on membrane translocation of small G proteins such as Ras and Rho, suggesting that statin anti-proliferative effect on macrophages is mainly attributed to the suppression of the GM-CSF-induced Ras and Rho-p38αMAPK signaling initiated by Ox-LDL.

In conclusion, the APC function can be down-regulated by statins at various levels: (1) antigen presentation, (2) adhesion with T-cells during antigen presentation, (3) costimulation, (4) activation and proliferation, and (5)

migration at the site of inflammation. These effects are more or less evident depending on the type of statin as well as on the type of APC.

Effects of statins on T-cells

Statins can have differential effects on T-cells depending on their structure, dose, and route of administration. In healthy humans, administration of 20 mg/day of atorvastatin for 14 days led to down-regulation of MHC II and CD38 activation marker on peripheral T lymphocytes, whereas treatment with 40 mg/day of simvastatin for 14 days upregulated both MHC II and CD38 molecules on T-cells [31]. Apparently, these contrasting effects of atorvastatin in particular, on MHC II and CD38 expression, are dose dependent.

Among all statins, fluvastatin and lovastatin were reported to exhibit the strongest suppressogenic effect on T-cells. Regardless of cholesterol reduction, oral administration of fluvastatin for 3 weeks in Lewis rats inhibited T-cell responses through inactivation of NF-κB transcription factor [32]. Also, fluvastatin was shown to reduce up to 90% the proliferation of human lymphocytes in response to CD3 stimulation [33]. The inhibitory effect of fluvastatin occurred in the context of a reduced functional activity of Ras-dependent extracellular signal-regulated kinase pathways, and Rho-dependent p38α MAPK kinase activation. Fluvastatin-induced suppression of T-cell proliferation was prevented by the addition of mevalonate or a farnesyl transferase inhibitor. First, these data revealed an HMG CoA reductase-dependent mechanism of T-cell suppression by fluvastatin, and second, they suggest that the drug inhibits subcellular pathways mediated by isoprenylation of signal peptides, including Ras, Rho, and related G-proteins.

Another mechanism of T-cell suppression is targeting the non-TCR (alternate) pathway of activation, i.e., intracellular calcium mobilization, inositol phosphate production and tyrosine phosphorylation of PKCγ1 and downstream events such as MAPK phosphorylation. Lovastatin blocked these proximal events in T-cells leading to suppression [34]. As these events occur independently of the TCR-mediated Ras signaling pathway, the suppressogenic effect of lovastatin could be explained by uncoupling of this alternative pathway of cell activation and proliferation.

Lovastatin was also reported to inhibit T-cell proliferation by suppression of cycle progression [35,36]. At equimolar concentrations, simvastatin and pravastatin suppressed T-cell proliferation, but to a lesser extent than Lovastatin [37]. Lovastatin-, simvastatin-, and pravastatin-induced T-cell suppression was prevented by the addition of mevalonate. The cell cycle progression involves activation of several kinases among which PKC. The HMG CoA reductase expression was found upregulated in CD3-stimulated T cells by a PKC-dependent pathway [36]. As the HMG CoA reductase generates mevalonic acid and its metabolites, data from the above studies suggest that one or more of mevalonate metabolites play critical role in cell cycle progression in T cells. The mevalonate pathway generates isoprenoids like geranylgeranyl and farnesyl moieties vital for various cellular function, including cell growth [38]. Thus, it has been shown that growth-regulating p21-ras proteins encoding for Ras proto-oncogenes and oncogenes, requires covalent farnesylation for anchoring to the plasma membrane [39–41]. There is a body of evidence showing that inhibition of mevalonate synthesis (by statin-mediated inhibition of HMG CoA reductase) prevents farnesylation of these proteins [39–44] with a subsequent block of cell growth [45]. Early studies showed indeed that HMG CoA reductase-dependent synthesis of cholesterol is also important for IL-2-mediated DNA synthesis in T cells (a major growth factor for T-cells), since inhibition of HMG CoA reductase by compactin (a strong competitive inhibitor) suppressed IL-2-mediated DNA synthesis and subsequently T cell growth [46].

Another mechanism of cell suppression by lovastatin pro-drug (-lactone ring) refers to the HMG CoA reductase-independent inhibition of proteosome degradation of p21 and p27 cell cycle inhibitors [47]. The lovastatin pro-drug does not inhibit the HMG CoA reductase, but it does inhibit the proteosome-mediated degradation of p21 and p27 cell cycle inhibitors. The addition of mevalonate abrogated the lovastatin pro-drug induced cell arrest in the G1 phase, suggesting that the mevalonate itself or one of its metabolites increases the activity of proteosomes leading to intracellular degradation of p21 and p27 cell cycle inhibitors.

Atorvastatin and simvastatin could also suppress T-cell cytotoxicity through down-regulation of FasL expression on the cell surface [48]. It is well know that Fas-FasL interaction is a major mechanism of T-cell death. Mevalonate and geranylgeranylpyrophosphate but not farnesylpyrophosphate prevented the suppressive effect of atorvastatin and simvastatin on T-cell cytotoxicity. This indicated first, that atorvastatin and simvastatin suppressogenic effect is HMG CoA reductase dependent, and second, that geranylation but not farnesylation is a critical event for FasL expression. Since RhoA is known to increase FasL expression, and RhoA is actively involved in protein prenylation, it has been suggested that the mechanism underlying atorvastatin and simvastastin suppression of FasL may well rely on FasL prenylation.

Fas receptor, a member of tumor necrosis factor family, is expressed on T-cells, and its expression is increased upon cell activation. It has been shown that mevastatin leads to spontaneous clustering of Fas receptor in skin cells leading to apoptosis following enhanced association of Fas with its signaling molecule FADD and caspase 8 [49]. Apoptosis in these cells occurred in the absence of FasL, and thereby the authors hypothesized that statins may induce spontaneous FasL-independent apoptosis in certain types of cells by a mechanism relying on cholesterol dependent re-distribution of Fas on cell surface with subsequent concentration in plasma cell membrane. Mevastatin also induced apoptosis of myeloma cells (U266 cell line) through the increase of caspase activity and depolarization of mitochondrial membrane, as well as repression of BcL-2 anti-apoptotic message. This apoptotic mechanism was not dependent on death-ligand/death-receptor pathway involving TRAIL, TNFR or Fas receptors [50].

Would alteration of these signaling events by statins lead to apoptosis in T-cells? Several reports indicated that cerivastatin, lovastatin, atorvastatin, simvastatin, and pravastatin do not induce apoptosis in T-cells [29,51–53].

Suppression of T-cells by atorvastatin was shown to occur by accumulation of p27 cell cycle inhibitor and a sustained phosphorylation of ERK1 [54]. Also, treatment of natural

killer (NK) T cells with lovastatin significantly suppressed their toxic effect on erythroleukemic target cells, and the suppression was abrogated by mevalonate [55]. Interestingly, IL-2 prevented lovastatin-induced suppression of these NK cells without affecting cholesterol biosynthesis or HMG CoA reductase pathway [56]. At higher doses in vitro (10 μM), lovastatin suppressed the NK T-cell cytotoxicity by more than 90%, and the phytohemagglutin- and concavalin A-induced T-cell proliferation by more than 80 and 30%, respectively [35]. These observations raised the concern of possible alteration of lymphocyte functions in patients undergoing prolonged therapy with statins. However, Peterson et al. demonstrated in a large number of patients with hypercholesterolemia undergoing lovastatin therapy (20 mg/day) for 8 weeks continuously that the number and function of T cells, B-cells, NK cells, as well as the level of immunoglobulins were not significantly reduced as compared to a placebo group [57]. One may consider that differences between the in vitro and in vivo assays may relate not only to the concentration of drug per se but also to the type of cell microenvironment in which statins exert their effects.

Perhaps, the most studied effect of statins-mediated protection in autoimmune diseases is Th1/Th2 immune deviation. The CD4 Th1 response is pathogenic, while the CD4 Th2 response is protective in most organ-specific autoimmune diseases. Polarization of the immune response towards a Th2 anti-inflammatory response by statins was observed in several animal models for autoimmune diseases and in T-cell cultures. Thus, low doses of lovastatin administered to SJL/J mice induced expression of GATA-3 and phosphorylation of STAT6 (transcription factors involved in T2 differentiation) whilst inhibiting phosphorylation of Jak2, STAT4, T-bet, and NF-κB (involved in Th1 differentiation) [61]. As expected, these mice showed a stabilized production of IL-4 and lower levels of IFN-γ. Also, atorvastatin was found to shift the polyspecific T-cell response towards a Th2 phenotype in humans and in mice [58–60]. Studies by Hakamada-Taguchi et al. revealed that among cerivastatin, simvastatin, lovastatin, atorvastatin, and pravastatin, the cerivastatin was the most potent statin in altering the Th1/Th2 balance towards a Th2 phenotype [51]. Pravastatin did not affect the Th1/Th2 balance, and this was correlated with its hydrophilicity, that could presumably impede the drug penetration through the cell membrane. Statin-induced Th2 deviation occurred mostly by Th1 suppression and less by Th2 augmentation regardless of genetic background. The Th2 shift was HMG CoA reductase-dependent, since it was abrogated by mevalonate. Finally, the authors showed that the Th2 shift induced by statins occurred in the presence or absence of APC, indicating a direct effect on T-cells.

As described above, it seems that some statins alter the T-cell function by a dual mechanism: Th1 suppression and Th2 deviation. This dual mechanism is supported by Affymetrix gene microarray data showing a differential repression vs. up-regulation of immune-related genes in T-cells treated with lovastatin. Thus, some 140 genes encoding for leukocyte specific markers and receptors, MHC, cytokine receptors, chemokine receptors, adhesion molecules, and components of cellular activation were repressed, while only 14 genes were up-regulated, i.e., peroxisome proliferators-activator receptor γ and the Th2 cytokine genes IL-4, IL-10, and TGF1 [61].

A key step in pathogenesis of inflammation mediated by lymphocytes is their ability to migrate and adhere to endothelial cells. Lovastatin was shown to inhibit migration of lymphocytes through the brain endothelial cells in a Rho-dependent manner [62]. Lovastatin also decreased tissue infiltration with lymphocytes by inhibiting expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin inducible by activation of PI-3K kinase/PKB (Akt)/NF-κB pathway in endothelial cells [63].

Some of the statin-mediated regulatory mechanisms leading to altered signaling expression of protein receptors critical for cell activation and proliferation of APCs and T-cells were attributed to the integrity of lipid rafts in plasma membrane. The lipid rafts are insoluble microdomains of plasma membrane made of cholesterol and glycosphingolipids [64,65]. These microdomains allow segregation of protein receptors through the lipid tail of glycosylphosphatidylinositol (GPI) leading to receptor compartmentalization into macromolecular signaling modules named signalosomes [66] The lipid rafts are essential for signaling of T and B-cells, and APCs [67–69]. Recently, it has been shown that simvastatin down-regulates expression of MHC class II and class I, CD19, CD40, CD80, CD83, CD86, and CD54 on APCs, as well as the expression of CD3 CD4, CD8, and CD28, on T-cells by disrupting the lipid rafts [70]. Simvastatin did not alter expression of CD45 molecules that usually do not segregate with lipid rafts in T-cells. Evidently, disruption of lipid rafts by statins may alter not only the antigen presentation by APCs, but also T-cell activation and differentiation with subsequent alteration of cytokine production and cytotoxicity. We found that lovastatin-mediated inhibition of HMG CoA reductase led to a decreased content of cholesterol in plasma membrane of T-cells, and this was associated with a Th1 predominant response in vitro [Brumeanu et al., unpublished results].

Disruption of lipid rafts by lovastatin was also associated with decreased expression and shedding of CD30 activation marker on lymphocytes by a mechanism of increased activity of TNFα-converting enzyme (TACE) residing in non lipid rafts microdomains [71]. Soluble CD30 was shown to suppress the Th1 response. Disruption of lipid rafts by statins can also lead to impairment of CD43 redistribution in plasma membrane with a subsequent decrease in T-cell motility [72].

Data presented in this chapter indicated that modulation of T-cell function by statins occurs by several complex mechanisms. The lipid rafts play a critical role in cell signaling, and their synthesis and composition depend on endogenous production of cholesterol controlled by HMG CoA reductase. It is likely that the mechanisms by which statins (as inhibitors of HMG CoA reductase pathway) induce alterations of APC and T-cell functions can involve the lipid rafts microdomains in plasma membrane.

Suppression of autoimmune responses by statins

Suppression of APC and T-cell function by reduction of MHC class II expression, inhibition of motility or expression of costimulatory ligands, or induction of T-cell hyporesponsiveness and immune deviation by statins has found several therapeutic applications in organ transplantation and auto-

immune diseases. In these pathological conditions, down-regulation of a polyspecific, self-reactive T-cell response is a desirable therapeutic approach. Statin effects were observed in several autoimmune diseases.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system in which the lesions in the brain and spinal cord are mediated by inflammation leading to demyelination, axon loss, and gliosis. Recent studies revealed autoimmune patterns of demyelination involving T cells and macrophages, as well as antibody/complement as effector mechanisms. Treatment of MS has advanced in recent years, with the introduction of beta-interferons, glatiramer acetate and mitoxantrone. However, not all MS patients respond well to treatment with these drugs, most likely due to disease heterogeneity. Statin therapy was recently investigated in MS patients and in experimental autoimmune encephalomyelitis (EAE), and proven to have down-regulatory effects on several key mechanisms involved in MS pathogenesis. Thus, atorvastatin was able to reverse paralysis in the central nervous system of EAE mice mainly by a Th2 anti-inflammatory immune deviation. Down-regulatory effects of atorvastatin on the immune function, i.e., inhibition of MHC class II, CD40, CD80, CD86 expression, suppression of IFN-γ secretion, interference with leukocyte migration and induction of apoptosis in Th1 inflammatory cells, were also demonstrated in the EAE model [29]. Lovastatin could also attenuate EAE by protecting the peroxisomal function in central nervous tissue through protection of catalase and DHAP-AT transcripts [73] and inhibition of Rho-mediated transendothelial migration of T-cells in central nervous system [62]. These studies demonstrated that statins like atorvastatin and lovastatin exert neuroprotective effects, which provides rational grounds for designing of new immune therapeutic strategies in MS (reviewed in ref. [74]). Statins are now being studied as therapeutics in MS and Clinically Isolated Syndrome.

Rheumatoid arthritis (RA) is a systemic inflammatory polyarthritis that destroys the synovial joints, and is associated with high prevalence of heart disease and cardiovascular mortality. The T and B-cell autoimmune responses against collagen in synovial joints plays an important role in the pathology of RA. The RA patients frequently show accelerated atherosclerosis [75], which was correlated with endothelial dysfunction due to persistent inflammatory damage. The beneficial effect of statins in RA refers to their anti-inflammatory capacity, inhibition of lymphocyte migration, adhesion, and growth, as well as inhibition of nitric oxide bioavailability in the milieu [76–78]. Simvastatin was shown to improve the endothelial function [79]. However, in a model for human rheumatoid arthritis, a short-term administration of either simvastatin, atorvastatin, or rosuvastatin showed minimal anti-arthritic activity as indicated by the clinical and histological parameters (synovial hyperplasia, exudate, cartilage damage), and immunological and biochemical parameters (anti-type II collagen IgG production, and IL-6, amyloid A, and glucocorticoid production) [80]. In contrast, long-term administration of 20 mg Atorvastatin per day for 12 weeks significantly reduced arterial stiffness in RA patients, although minimal changes in serum inflammatory markers were observed [81]. A large clinical trial on 116 RA patients treated for 6 months with higher dose of atorvastatin (40 mg/day) showed that the disease activity variables and circulating vascular risk factors were significantly improved in 95% patients [82]. Similar outcomes were obtained in patients with different inflammatory rheumatic diseases refractory to conventional therapy when treated with simvastatin (80 mg/day) for only 8 days [83]. The validation of simvastatin potential therapeutic effect was demonstrated in a Th1-driven model of murine inflammatory arthritis, in which the drug was used in doses smaller that those required for reduction of blood cholesterol levels [53]. It thus appears that the benefit of statins therapy in RA is promising but still needs to be investigated, particularly in relation to the type of statins and regimen of administration.

Systemic lupus erythematosus (SLE) is a multisystem disease characterized by a wide variety of immunological disorders. Atorvastatin was reported to inhibit B-cell secretion of anti-dsDNA IgG antibodies and decrease the immunoglobulin deposition in kidney with subsequent improvement of glomerular function in a mouse model for lupus [84]. The disease progression was significantly reduced in these mice. This was associated with reduced polyclonal T-cell response in the context of inhibition of CD80 and CD86 expression on B-cells and monocytes. Interestingly, a lupus-like syndrome was reported in animals treated with fluvastatin and simvastatin [85,86]. Initially, these observations suggest that the beneficial effect of statins in lupus may depend on their chemical structure.

An undergoing clinical trial is carried out at National Institutes of Health (Bethesda, USA) using atorvastatin (lipitor) in children with SLE. Children and adolescents with SLE represent 15% of all SLE patients. Children with SLE suffer high morbidity affecting many organs, and their lifespan is significantly reduced. As these children survive into adulthood, atherosclerotic cardiovascular disease has emerged as a major concern. SLE is also a significant risk factor for myocardial infarction and death in young premenopausal women with SLE. Acceleration of atherogenesis in SLE most likely reflects SLE-associated vascular immune and inflammatory changes.

Statins have shown to reduce mortality and morbidity from atherosclerosis in adults by anti-inflammatory effects and immune modulatory properties. These anti-inflammatory and immune modulatory activities may have particular benefit in the prevention and treatment of atherosclerosis in SLE. This trial will evaluate atorvastatin in children with SLE in a large cohort of pediatric SLE patients.

The therapeutic effects of statins were tested in several other autoimmune animal models. Thus, autoimmune myocarditis induced in Lewis rats by immunization with myosin was attenuated after 3 weeks administration of fluvastatin [32]. The therapeutic effect was associated with inhibition of T-cell responses, particularly of Th1 response, and suppression of inflammatory cytokines in myocardium via inactivation of NF-κB. These effects occurred independently of fluvastatin-mediated cholesterol reduction. The apoptotic effect of statins like simvastatin on the infiltrating lymphocytes was found beneficial in patients with autoimmune Hashimoto's thyroiditis, since their thyroid functions, i.e., TSH levels, were significantly improved [87]. In contrast to atorvastatin, lovastatin suppressed the ocular

pathology, retinal vascular leakage, and lymphocyte infiltration into the retina in mouse models of autoimmune retinal disease [88] as well as in autoimmune uveitis [89]. The therapeutic benefit of lovastatin were correlated with the drug ability to inhibit Rho GTPases-dependent prenylation of ICAM-1, an adhesion molecule with a role in lymphocyte migration, rather than to a Th2 immune deviation. In patients with primary biliary cirrhosis, an autoimmune disease of the liver with inflammation of small and middle-sized bile ducts and high titer of antimitochondrial antibodies (AMA), simvastatin treatment led not only to reduction of cholestasis, but also lowered AMA titer [90]. These observations highlighted a differential activity of statins in different autoimmune conditions.

Prevention of type 1 diabetes by atorvastatin (lipitor)

Type 1 Diabetes (T1D, Insulin-dependent diabetes mellitus, IDDM) is a chronic autoimmune disease resulting from T-cell-mediated destruction of pancreatic -cells [91]. To date, the autoimmune phenomena in T1D has been the most studied. In humans as well as in non-obese diabetic (NOD) mice, susceptibility to the disease is conferred by the expression of particular MHC class-II alleles and non-MHC class II allele [92]. Epidemiological data demonstrating geographical differences in the incidence and prevalence of IDDM [93], age of onset [94], seasonally [95,96], and discordance for diabetes in monozygotic twins [97] indicate that environmental factors are also involved in the pathogenesis of IDDM. Among these, infectious, chemicals, and dietary factors are prominently involved, although the underlying mechanisms by which these factors contribute to the disease are still unclear [98]. The genetic background and environmental factors lead to the initiation of an autoimmune process against the pancreatic -cells, culminating in -cell destruction and loss of insulin secretion.

In humans and animal models for spontaneous autoimmune diabetes, the T-cell reactivity against -cell antigens, i.e., glutamic acid decarboxylase 65 (GAD65) [99], insulin [100], tyrosine phosphatase IA-2 [101], heat shock protein 60 (hsp60) [102], and the islet-cell antigen 69 (ICA69) [103] have been demonstrated. The pathogenicity of CD4 T-cells in diabetes is closely related to the Th1 cells, while protection is associated with Th2 and CD4 T regulatory cells [91,104].

We have used a TCR-HA/RIP-HA double transgenic (dTg) mouse model for T1D to test the effect of atorvastatin on the course of disease. The TCR-HA/RIP-HA dTg mice express the hemagglutinin protein (HA) of PR8 influenza virus in pancreas within the rat insulin receptor (RIP-HA), and at the same time an HA110-120 specific T cell receptor (TCR)-HA) on T-cells. We previously showed that these mice develop pancreatic infiltration with lymphocytes some 5 days after birth, and hyperglycemia 30–35 after birth [105]. Regardless the gender, some of these mice develop an aggressive T1D and have a short life (2–3 months), whereas some mice develop a milder form of T1D and live longer (4–5 months). The hyperglycemia in mice with aggressive T1D can be detected as early as 4–5 weeks after birth, whereas in those developing the mild form of disease is detected 7–8 weeks after birth.

Fourteen day-old prediabetic TCR-HA/RIP-HA dTg newborns (n = 12) were treated orally with Atorvastatin (Lipitor in saline, 10 mg per Kg of body weight) for 14 days, every other day. Glycemia was measured by-weekly, starting 1 week after beginning of the treatment. Some 80% of mice (n = 10) were euglycemic for 7 months after interruption of treatment, whereas the dTg control mice (treated with saline) developed hyperglycemia by day 30–35 after birth and survived only 4–5 months (Fig. 2). The pancreas of protected mice analyzed 1 week after interruption of treatment was free of lymphocyte infiltration, and showed normal levels of intra-islet insulin. In contrast, the control mice showed hyperglycemia higher than 600 mg/dl, massive β-islet infiltration with lymphocytes, and poor intra-islet secretion of insulin (Fig. 3).

Further analysis of the protected mice showed a Th2 prominent phenotype in spleen 1 week after interruption of treatment, whereas the diabetic mice showed a dominant Th1 response (Table 1). However, there was no significant alteration in the proliferative capacity of CD4 T-cells upon in vitro polyclonal stimulation with ConA in atorvastatin-treated mice as compared with the control group. Several transcription factors involved in Th1 and Th2 differentiation were analyzed 1 week after interruption of treatment in negatively-immunosorted, splenic CD4 T-cells by RT-PCR. The results indicated an increase in the mRNA levels for STAT6, cMAF, and GATA3 (Th2 transcription factors) in mice protected by atorvastatin, whereas the diabetic ones showed higher expression level of mRNA for STAT4 and T-bet (Th1 transcription factors (Fig. 4).

Using the same regimen of administration, we have also tested the effect of atorvastatin (lipitor) in already hyperglycemic dTg mice. Interesting enough, 40% of those mice (n = 4) developing the mild form of T1D showed

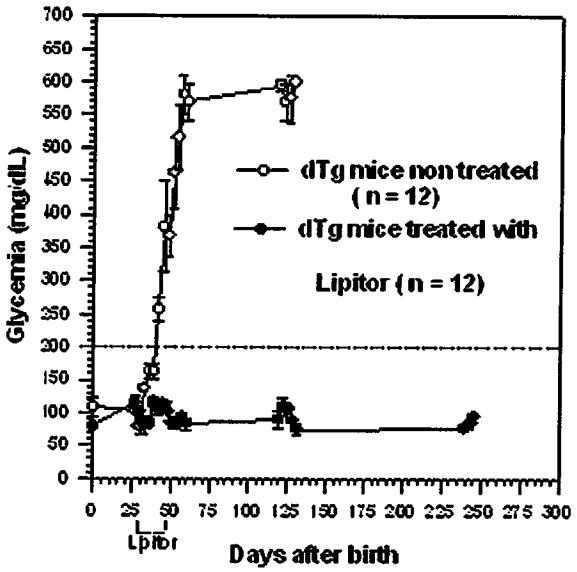

Figure 2 Glycemia levels in dTg mice treated with atorvastatin (lipitor). Shown are the Atorvastatin treated mice (n = 12 ± S.D.) vs. those untreated. The doted line indicates the upper limit of normoglycemia as determined in a cohort of 21 Balb/c mice after 12-h fasting.

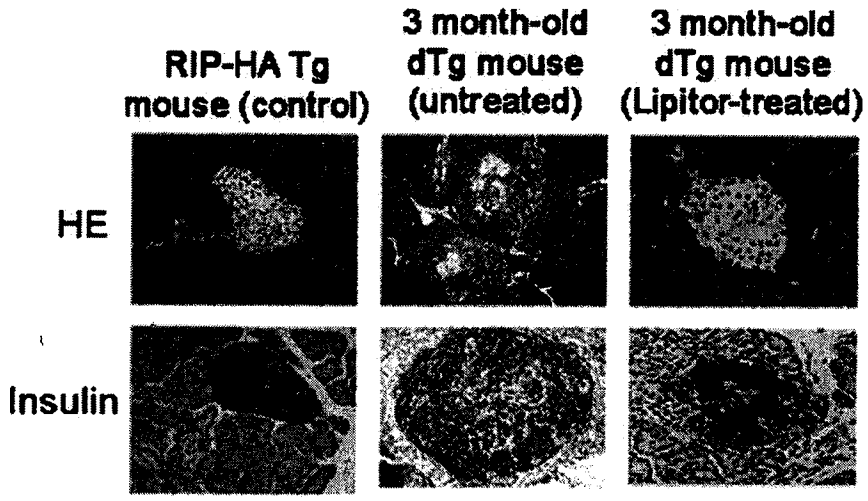

Figure 3 Histopathology of pancreata in dTg mice protected by atorvastatin (lipitor). Shown are cross-sections of the pancreas from dTg mice treated (or not) with atorvastatin. Upper panels, hematoxylin–eosin stain. Lower panel, intra-islet staining of insulin with a rabbit anti-insulin-HRP conjugate at 200× magnification. Lymphocyte infiltrates of the islet were detected only in the untreated dTg mouse. As shown, the intra-islet secretion in the untreated mouse is significantly lower than in the control and atorvastatin-protected mouse.

stabilized levels of glucose in blood (450–500 mg/dl) and 1 mouse out of 10 showed a normal level of glycemia (138 mg/dl) after only 3 oral doses of lipitor. In contrast, under the same regimen of administration, hyperglycemia was not affected in mice developing the aggressive form of T1D. The anti-diabetogenic effect of lipitor is currently investigated in our laboratory in NOD female mice at various stages of disease. Although the experiments in hyperglycemic dTg mice did not reach the biostatistic relevance yet, one may assume at this very moment that atorvastatin therapeutic effect could be more beneficial in prevention, before, and at the onset of diseases, than in a more advanced stage of disease.

As in humans with T1D and in NOD mouse model for human T1D, in the TCR-HA/RIP-HA dTg mice, the disease is driven by Th1 cells. Our data indicated for the first time that HMG CoA reductase inhibitors like atorvastatin can down-regulate the function of diabetogenic CD4 T-cells by means of Th1/Th2 deviation. At present, we have not investigated whether the Th1/Th2 deviation in dTg mice is the only mechanism of protection, and whether atorvastatin-induced Th2 deviation depends solely on the inhibition of HMG CoA reductase.

Our data raised the question whether the anti-diabetogenic effect of atorvastatin in these mice may contribute to improvement in pancreatic β-cell function, in addition to its regulatory effect on T-cells. At first, some data in the literature indicated that inhibition of prenylation by lova-

Table 1 Cytokine secretion by splenic cells from dTg mice protected by atorvastatin (lipitor)

Cytokines	3-month-old dTg mouse (untreated) (pg/ml ± S.D.)	3-month-old dTg mouse (Lipitor-treated)
IL-2	920 ± 57	415 ± 7.2
IL-4	112 ± 40	828 ± 10.1
IFN-γ	1.250 ± 8.7	651 ± 3.5

Cytokines were determined by ELISA in a 3-day culture in the presence of CD3/CD28 polyclonal stimulation. Values represent the cytokine levels in the cell culture supernatants ± S.D. (triplicate wells for intra-assay variation). Similar values were obtained for other 3 dTg mice protected by atorvastatin.

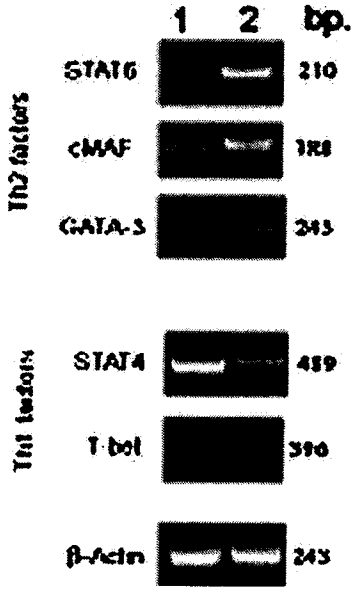

Figure 4 Expression of Th1 and Th2 transcription factors in dTg mice protected by atorvastatin (lipitor). Lane 1, a 3-month-old diabetic (untreated) dTg mouse. CD4 T-cells were isolated from spleen of mice treated or not with atorvastatin, and RNA extracted and RT-PCR carried out using specific primers as we previously described [112]. Lane 2, a 3-month-old atorvastatin-treated dTg mouse. Similar results were obtained in other 3 dTg mice protected by atorvastatin.

statin in normal rat islets reduced the glucose-induced insulin secretion by 50% [106]. This was associated with accumulation in the cytosolic compartment of small GTP-binding proteins involved in insulin secretion due to a deficient isoprenylation. It has been also reported that lipophilic statins like simvastatin, but not the hydrophilic ones like pravastatin, can inhibit glucose-induced cytosolic Ca^{2+} and insulin secretion in normal rat β-islets by a blockade of the L type Ca2+ channels [107].

Several beneficial effects of statins on the pancreatic β-islets have been also described. Thus, pravastatin improved the insulin secretion by β-islets transplanted in dogs with pancreatectomy for 12 weeks, and considerably prolonged their survival. This was associated with a reduced nonspecific periislet inflammation [108]. Nonspecific inflammation is a major cause of early loss of β-islets grafts. Also, lovastatin showed an anti-fibrotic effect on pancreas, since it was able to inhibit the serum-, and platelet-derived growth factor-stimulated pancreatic stellate cell activation and proliferation [109]. The pancreatic stellate cells (PSC) play an important role in developing pancreatic fibrosis. The PSC are activated by pro-inflammatory cytokines by a Ras–Raf–ERK signaling pathway towards a myofibroblastic phenotype with increased secretion of extracellular matrix proteins. The authors suggest that the anti-fibrinogenic mechanism of lovastatin may rely on HMG CoA reductase inhibition with subsequent inhibition of isoprenylation required for activation of Ras–Rasf–ERK pathway of PSC activation and proliferation. Furthermore, fluvastatin was shown to protect against the oxidative damage of pancreas that occurs by formation of reactive oxygen species [110], and simvastatin was reported to exert protection against pancreatic β-cells apoptosis through activation of PI-3K/Akt signaling pathway and decreased phosphorylation (activation) of Bad pro-apoptotic protein [111].

In summary, the data reported in literature refer mostly to the prevention and symptoms amelioration in several organ-specific autoimmune diseases, i.e., multiple sclerosis, rheumatoid arthritis, autoimmune myocarditis, autoimmune uveitis, autoimmune Hashimoto's thyroiditis, and systemic lupus erythematosus. Our data indicated that atorvastatin (lipitor) had therapeutic effects in mice with type 1 diabetes, particularly when administered before the onset of disease. With the exception of atorvastatin, which induces a shift from Th1 to Th2 cell response, the predominant therapeutic effect o other statins refers to the suppression of Th1 anti-inflammatory response, apoptosis, or anergy of the autoreactive lymphocytes. Statins exert pleiotropic effects depending not only on the cell type, but also on the type of statin. At the same time, a particular statin may interfere with various signaling mechanisms in a given cell type. Taking into account these considerations, one may achieve optimal therapeutic effects in autoimmune diseases and grafts transplantation by considering 3 major parameters: the type of cell target, the type of statin, and the regimen of administration. One might also want to consider that, although some statins may not exert strong regulatory effects directly on autoreactive T-cells, they may efficiently down-regulate the inflammatory reactions of APCs and other cell types responsible for systemic complications like in overtly autoimmune diseases.

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Development of Novel Compounds to Treat Autoimmune and Inflammatory Diseases and Graft Versus Host Reactions

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Abstract: Recently, several new classes of agents were developed to treat patients with malignant diseases. This progress has been based on the advances made in our understanding of critical pathways involved in tumor development and growth. Dysregulated processes leading to uncontrolled regulation of proliferation, cell cycle progression, angiogenesis and apoptosis have provided rational targets for novel therapies. Compounds inhibiting protein phosphorylation and signal transduction like tyrosine kinase inhibitors and inhibitors of proteasomal degradation have demonstrated promising results and were approved for the treatment of patients with malignant diseases. However, based on *in vitro* and *in vivo* studies, there is now an emerging evidence that these agents can affect the function and differentiation of normal, non-malignant cells like dendritic cells or T lymphocytes, resulting in immunosuppression. In our review we present recent data on the immune regulatory effects of tyrosine kinase inhibitors like imatinib that is approved to treat chronic myeloid leukemias, or inhibitors of FLT3, currently used to treat acute leukemias, as well as proteasome inhibitors and peroxisome proliferator-activated receptor agonists and discuss their possible role and application in the treatment of autoimmune and graft versus host disease.

Key Words: Tyrosine kinase inhibitors, proteasome, autoimmunity, graft versus host, immunosuppression.

IMMUNE REGULATORY EFFECTS OF TYROSINE KINASE INHIBITORS

In the last years, progress has been made in the development of new targeted cancer therapies using compounds like tyrosine kinase inhibitors that affect signal transduction pathways involved in tumor growth or angiogenesis.

One of these drugs, imatinib (Gleevec), is an inhibitor of the Abl protein tyrosine kinases (v-ABL, BCR-ABL, and c-ABL) [1-5], platelet-derived growth-factor receptor (PDGF-R) [6, 7], c-KIT (CD117) [8], Abl related gene (ARG) [9] and macrophage colony stimulating factor receptor (c-fms) [10]. It is approved for the treatment of chronic myeloid leukemia (CML), and in many patients, complete hematological and cytogenetic responses could be induced [2, 3, 11-13]. Imatinib is also active in accelerated phase or blast crises CML and in patients with primary, relapsed or refractory Philadelphia chromosome positive (Ph^+) acute lymphoid leukemias (ALL) [14, 15]. Moreover, imatinib is used in the treatment of patients with gastrointestinal stroma tumors (GIST) caused by activating mutations of c-KIT or PDGF-Rα [16-18] and myeloproliferative disorders with mutations in the gene encoding PDGF-R [19-21].

The moderate hematological side effects observed with imatinib therapy are dose-dependent and reversible, and analyses with GIST patients suggested that they are caused at least in part by direct inhibition of normal hematopoiesis [16, 22]. Further investigations revealed that imatinib indeed not only acts on malignant cells, but also affects the function of normal, non-malignant cells. Although not affecting stem cells, imatinib treatment decreased the expansion of normal CD34+ progenitor cells [23]. Dewar and colleagues showed that imatinib inhibits the development of the monocyte/macrophage lineage from CD34+ progenitor cells *in vitro* and also impedes the differentiation of monocytes into macrophages [24]. Moreover, they showed that the functional capacity of cultured monocytes is impaired [25]. Studies with dendritic cells (DCs) revealed that their differentiation from CD34+ progenitor cells as well as monocytes is affected in the presence of imatinib [26, 27]. The drug reduces the capacity of DCs to stimulate T cells [26, 27]. These inhibitory effects of imatinib on DC development and function were further confirmed *in vivo* in a mouse model [28]. Feeding of animals with imatinib resulted in a reduced number of DCs as well as impaired protective anti-tumor responses [28]. These effects are at least in part mediated *via* inhibition of RelB signaling, a member of the NF-κB transcription factor family [26, 27]. Interestingly, imatinib-treated DCs were able to activate NK cell effector functions *in vitro* and *in vivo*, thereby providing a novel anti-tumor activity of the drug [29]. Imatinib was also shown to impede the proliferation [30] and activation [31] of T cells by inhibiting early signaling *via* the T cell receptor [32], resulting in reduced interleukin-2 production by the activated T cells [32].

Interestingly, regression of type II diabetes was reported in patients treated with imatinib [33, 34]. Moreover, two studies in mice have demonstrated that imatinib treatment reduced diabetes-associated atherosclerosis [35] and diabetic nephropathy [36], indicating that PDGF-dependent pathways might be involved in these conditions, providing a target for imatinib.

In a murine model of systemic lupus erythematosus (SLE), a systemic autoimmune disease, imatinib treatment improved the progressive inflammatory glomerular disease and sur-

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vival of treated animals [37, 38]. Overexpression of PDGF has been assumed to be involved in disease progression. However, inhibition of immunological and inflammatory processes might also play an important role.

FLT3 receptor tyrosine kinase represents a novel possible target for the development of new cancer therapies. Increased levels of FLT3 are associated with leukemia and mutations leading to constitutively active FLT3 signaling, the most common genetic alterations in acute myeloid leukemia (AML) patients, are associated with a poor prognosis [39-41]. Therefore, several FLT3 inhibitors have been developed for the treatment of AML that are currently analyzed in clinical trials [42-48].

In several *in vitro* and *in vivo* models, it has been demonstrated that the presence of FLT3 ligand (FLT3L) can mediate the development of both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) from CD34+ progenitor cells and plays a central role in the differentiation of DCs from early progenitor cells [49-52]. In a recent study, Tussiwand and co-workers showed that the receptor tyrosine kinase inhibitor SU11657, which is active against FLT3, vascular endothelial growth factor receptor (VEGF-R), PDGF-R and c-KIT, inhibits pDC development in mice *in vitro* and *in vivo* [53].

These effects on DC development and function might be exploited for the therapy of diseases, where DC dysfunctions and/or activated T cells might contribute to the disease manifestation. DCs play a pivotal role in the induction of central as well as peripheral tolerance [54, 55]. It is therefore conceivable that dysfunctioning of DCs results in the presentation of autoantigens on mature DCs, leading to stimulation of autoreactive T cells instead of their anergy, resulting in autoimmunity. In several model systems, DCs have indeed been shown to induce and maintain autoimmune diseases [56, 57], indicating a crucial involvement of these cells in the development of autoimmunity. Defects in DC apoptosis might also be important in this regard. A recent report showed accumulation of DCs and induction of autoimmunity after blocking normal apoptosis of DCs [58].

Interestingly, dysregulation of pDCs was also suggested to be involved in the manifestation of autoimmune diseases like SLE and Sjögren's syndrome (SS), as those patients have an activated interferon-α system [59-62].

Inhibition of DCs with imatinib or compounds targeting the FLT3 system might therefore provide a helpful alternative for treatment of these patients. Furthermore, malignant diseases evolving from cells with DC phenotype like Langerhans histiocytosis may be suitable for such a therapeutic application.

Another interesting and promising therapeutic approach to exploit the inhibitory effects on DCs is the reduction of complications after transplantations where residual host DCs are involved in the initiation of graft versus host disease (GVHD) [63-67]. This alloimmune attack on host tissue is mounted by donor T cells after allogeneic bone marrow or mobilized blood stem cell transplantation. It primarily affects skin, gut and liver and is a major toxicity that limits the efficacy of this treatment. As skin is the organ most commonly affected in GVHD, several studies have been carried out targeting Langerhans cells (LCs), the major antigen-presenting cell population in the skin. Depletion of these cells before transplantation prevented GVHD in a mouse model [67] and in human *in vitro* systems [68, 69].

EFFECT OF PROTEASOME INHIBITORS ON NORMAL CELLS

Proteasome inhibitors like bortezomib have shown promising results in the treatment of various malignant diseases like multiple myeloma [70-73], non-Hodgkin lymphoma [74, 75], acute leukemia [76] and various solid tumors [77, 78]. However, the mode of action for their anti-cancer activity is not yet clarified, although NF-κB inhibition was shown to be involved [79, 80]. Several additional mechanisms have been proposed, including mainly the stabilization of pro-apoptotic as well as regulatory proteins, stabilization and accumulation of p53, and unbalances in the pro- and anti-apoptotic Bcl-2 family proteins [81, 82].

Members of the NF-κB family of transcription factors play a major role during inflammation and induction of immune responses. Several studies using bortezomib were performed to analyze possible advantageous effects by inhibiting these transcription factors in immune-mediated disorders. Accordingly, bortezomib treatment was shown to be an effective therapy in animal models for polyarthritis [83], psoriasis [84] and experimental autoimmune encephalomyelitis [85]. These beneficial effects might result from the inhibition of DC function and differentiation, as recent studies demonstrated that bortezomib induces apoptosis in monocyte derived DCs and interferes with DC function, most likely *via* the inhibition of the NF-κB pathway [86, 87].

Proteasomal degradation is essential for MHC class I restricted antigen presentation [88, 89]. Moreover, we could recently show that the proteasome is also required for the presentation of cytoplasmic proteins on MHC class II molecules [90]. This discloses interesting possibilities to use proteasome inhibitors in GVHD and immune disorders caused by activated T cells. Animal models have already shown promising results in treating GVHD [91, 92], although a prompt start of the treatment following bone marrow transplantation was essential [91]. Blanco and co-workers confirmed the advantageous GVHD treatment with bortezomib in an *in vitro* study using human peripheral blood mononuclear cells, as it selectively depleted alloreactive T lymphocytes and decreased T helper type 1 cell (Th1) cytokine production [93], whereas unstimulated T cells were barely affected. However, a recent study showed that even though bortezomib treatment initially prevented GVHD in a mouse model, extended therapy resulted in substantial GVHD-related mortality [94].

THE CONTROL OF IMMUNE RESPONSES BY PPARS AND ITS LIGANDS

The family of peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors involved in the regulation of metabolism and inflammation [95-97]. They are members of the nuclear hormone receptor superfamily and consist of PPAR-α, PPAR-β or –δ, and PPAR-γ. Upon ligand binding, they dimerize with the retinoid X receptor (RXR) and bind to PPAR response elements (PPRE)

in the promoter region of target genes. In addition to ligand-dependent activation, PPAR-γ is regulated by phosphorylation [97]. Several studies have shown that PPAR-γ activation negatively regulates pro-inflammatory transcription factors like NF-κB, NFAT, AP-1 and STAT [98-101]. Ligands of PPAR-γ include naturally occurring prostaglandin D2 (PGD_2) and its metabolite 15-deoxy-$\Delta^{12,14}$-PGJ2 (15d-PGJ_2), other cyclopentanone prostaglandins as well as synthetic thiazolidinediones.

Activation of PPAR-γ has been shown to impair the phenotype and immune-stimulatory function of DCs [102-104] and inhibit toll-like receptor-mediated activation of DCs, *via* MAP kinase and NF-κB pathways [105].

In animal models, PPAR-γ activation has been shown to have positive effects on several inflammatory diseases by modulating Th1/Th2 balance and reducing the expression of pro-inflammatory cytokines [106-114]. In inflammatory bowel disease, where epithelial cells of the colon produce inflammatory cytokines that may be involved in disease manifestation, activation of PPAR-γ negatively regulated this cytokine gene expression by inhibiting NF-κB activation [106-108]. Setoguchi and co-workers reported that haploinsufficiency of PPAR-γ in PPAR-$\gamma^{+/-}$ mice resulted in more severe antigen-induced arthritis [109], and consistent with that, Kawahito and co-workers found that PPAR-γ activation suppressed experimentally induced arthritis in rats [110]. PPAR-γ activation was also shown to be beneficial in experimental autoimmune myocarditis [111] and encephalomyelitis [112-114] by reducing the expression of inflammatory cytokines. Moreover, the PPAR-γ agonist rosiglitazone has shown promising results for the treatment of ulcerative colitis in preliminary clinical evaluations [115]. As agonists of PPAR-α have also been shown to shift the cytokine production of T cells *in vitro* from Th1 to Th2, they have been suggested for the treatment of inflammatory conditions such as multiple sclerosis [116, 117].

CONCLUDING REMARKS

Based on these preclinical *in vitro* and animal studies and first clinical observations, these new classes of compounds originally developed to treat malignant diseases might represent a novel therapeutic option for patients with autoimmune disorders like multiple sclerosis or inflammatory bowel diseases. Furthermore, they provide a promising approach to handle graft versus host reactions in patients receiving allogeneic bone or stem cell transplantation. The final significance and efficacy of these drugs have to be evaluated and confirmed in clinical trials.

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The number needed to treat for adalimumab, etanercept, and infliximab based on ACR50 response in three randomized controlled trials on established rheumatoid arthritis: a systematic literature review

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Objective: To compare the efficacy of adalimumab, etanercept, and infliximab in patients with established rheumatoid arthritis (RA) taking concomitant methotrexate (MTX) by calculating the number needed to treat (NNT) using three different methods.

Methods: A systematic literature search of the Cochrane Library, MEDLINE, and EMBASE was conducted from inception to 30 June 2006. Two pairs of investigators, a Danish and a Swedish pair, independently conducted a structured literature review. The reviewers selected any published randomized, double-blind, MTX controlled study of adalimumab, etanercept, and infliximab, presenting the American College of Rheumatology 50% response (ACR50) after 12 months in RA patients with a mean disease duration of at least 5 years. The two review groups independently extracted the estimates necessary to calculate the NNT.

Results: The reviewers consistently selected the same three randomized, controlled trials (RCTs), one for each of the drugs, and extracted equal data for the number of patients completing the 12-month intervention, and the corresponding number of ACR50 responding patients after therapy. Some baseline differences were noted: patients in the etanercept trial had a shorter disease duration and did not receive MTX prior to inclusion; patients in the adalimumab study had lower Health Assessment Questionnaire (HAQ) scores. The calculated NNTs varied slightly depending on the method used. The fully adjusted NNTs (95% confidence intervals) for adalimumab, etanercept, infliximab standard dosage and infliximab double dosage were 4 (3–6), 4 (3–6), 8 (4–66), and 4 (3–11) patients, respectively.

Conclusion: This study indicates equal efficacy of the three anti-tumour necrosis factor (TNF) therapies.

Evidence-based medicine is the application of the most valid scientific information in the care of patients. It is becoming increasingly important when providing clinicians with guidelines on how to treat a disease (1, 2). Decision making in medicine relies on updated reviews of medical research results. The challenge is to translate published clinical research data into a format suitable for practical application in the clinical setting, where the clinician must choose between competing interventions that have not been compared directly (head-to-head) in randomized trials (3–5).

In rheumatoid arthritis (RA), which is the most common inflammatory arthritis disorder (6), the armamentarium of interventions has increased considerably over the past 5 years (7). Tumour necrosis factor-α (TNFα) blocking drugs have been studied extensively and play a major role, both financially and clinically, in the current treatment of RA (7). TNFα is a proinflammatory cytokine important in RA development (7). Three TNFα blocking drugs have been registered: adalimumab, etanercept, and infliximab. However, direct comparisons of these drugs in randomized controlled trials (RCTs) are lacking. Cochrane reviews have been published separately for each of these drugs (8–10), and clinical decisions about which to use remain highly individual and lack the support of official guidelines (11).

An approach increasingly used for comparing drug responses is the 'number needed to treat' (NNT), the reciprocal of the absolute risk difference (12). This approach has also been recommended in rheumatology (13). The NNT has the advantage of being readily understood in a statistical setting and by the practitioner trying to grasp an overall measure of

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efficacy of a given drug (12–14). Provided the data allow a dichotomization, the NNT will give the reader a standard measure that can be used for indirect comparison of clinical efficacy across multiple RCTs testing different preparations in trials of similar duration (15). Three methods for calculating the NNT can be applied: among completers, among randomized patients (the usual way of calculating the NNT), and finally among randomized patients with a further adjustment for differences in event rates of the control group (16, 17). Other approaches to indirect treatment comparisons of RCTs are mixed treatment comparison and meta-regression analysis; however, these methods are complicated and still having shortcomings, and do not have the clinically informative value that the NNT does (18). Relative measures such as odds ratios have also been used for indirect comparison (19, 20), despite inherent limitations of relative measures, that is ignoring the absolute underlying chance of responses and thus being less applicable to a clinical situation (21). In addition, evidence may also come from observational studies, where a recently presented index, the LUNDEX, facilitates comparisons of interventions (22).

Using a structured protocol for search of, and retrieval from, the present literature, the objective of this systematic review was to compare NNTs based on the three different types of calculations for adalimumab, etanercept, and infliximab combined with methotrexate (MTX), from the published double-blind, 12-month RCTs in established RA. As the defined treatment response criterion we used the American College of Rheumatology 50% response (ACR50).

Material and methods

Literature review

The systematic review was conducted by two independent review groups: a Danish group consisting of RC and HB, and a Swedish group consisting of LEK and PG. The two review groups followed a predefined protocol: reviewers searched the Cochrane Musculoskeletal Group's Specialized Register of Controlled Clinical Trials. Only published material written in English was considered. The final literature search was primarily based on an alternative strategy using the systematic reviews performed by the Cochrane Musculoskeletal Group (CMSG) for each of the three TNFα blocking agents for RA. Because of limitations in recent updates of the CMSG reviews, reviewers also performed a complementary search using the Cochrane library, MEDLINE and EMBASE from inception to 30 June 2006. RA was searched as an exploded MeSH heading ('arthritis, rheumatoid' [MeSH Term] OR rheumatoid arthritis [Text Word]). The aim was to include studies of each registered TNFα blocking preparation (in alphabetical order): adalimumab, etanercept, and infliximab. Selection criteria were: publications presenting data from RA patients with an average disease duration of at least 5 years and explicit data on the ACR50 response after at least 12 months of follow-up; studies had to be double-blind RCTs with a minimum of two arms comparing the TNFα blocking preparation (intervention) and concomitant use of MTX compared to MTX alone (control). Twelve months of follow-up was chosen to give an expression of the long-term sustainability of TNFα blocking drugs, which are normally administered over several years.

The ACR50 response was chosen because a recent meta-analysis showed this to be the preferred endpoint for contemporary RA clinical trials compared to ACR20 (23) based on both statistical and clinical evidence. ACR20 or ACR50 responders are the number of patients achieving at least 20% or 50% disease reduction, respectively, after intervention (24). Because of the strict inclusion criteria and the size of the trials included, review of abstract databases was omitted from this review.

Data analyses: applying absolute and relative numbers

After selecting the articles, the two review groups independently extracted explicitly reported numbers (i.e. unbiased estimates) from the published, peer-reviewed material, making no assumptions other than those already explicitly presented in the articles. The predefined baseline characteristics required were age, percentage female, disease duration, percentage rheumatoid factor positive, prior MTX dose, C-reactive protein (CRP), Health Assessment Questionnaire (HAQ), number of tender joints and number of swollen joints, number of patients included, ACR50 response data at end point, and data on number of patients withdrawn at end point, for both the intervention and the control arm of the studies. For studies with more than one intervention arm, the arm containing the recommended standard dosage was chosen. Because infliximab is known to regularly require dosage adjustments, the arm containing standard as well as doubled dosage was selected for this preparation. Based on these numbers we were able to calculate the percentage responders both as a per-protocol (PP, completer analysis) and as intention-to-treat (ITT, non-responder analysis). Adherence to therapy was defined as patients attending the examination after intervention; accordingly, this number is synonymous to the PP population. Among the patients adhering to therapy, the estimated percentage of ACR50 responders among completers and randomized patients was calculated as ratios,

with the denominator being the number of completers (PP) and randomized patients (ITT), respectively. NNTs (the reciprocal of the absolute percent ACR50 responders) were calculated as two independent efficacy outcomes, among completers and randomized patients, respectively. Finally, a third method for calculating the NNT was performed by adjusting for differences in response rates of the control group. As the control event-adjusted NNT values are applicable only to an ITT population, according to the CONSORT statement (25), we calculated the odds ratio among ITT patients responding according to an ACR50 criterion, and converted this into NNTs (14, 16) with the %control group events as baseline risk (26) (www.nntonline.com).

Results

The study selection process performed by the two independent review groups was very similar. The Swedish and Danish reviewers agreed on the flow chart in Figure 1. During the initial search, 98 potentially relevant publications were retrieved. Of these, 86 were excluded on the basis of title and abstract review; 12 trials were assessed for inclusion and exclusion criteria (27–38). Of these, nine trials were excluded based on short disease duration, inadequate control/intervention groups, and lack of a 12-month follow-up (27–29, 31, 33–35, 37, 38). Consistently, the Swedish and Danish review groups independently found one publication fulfilling the criteria for each of the three compounds (30, 32, 36).

Demographic data and characteristics of patients enrolled in the three studies are summarized in Table 1. We noted some dissimilarities between the three studies: patients in the etanercept trial had shorter disease duration than patients in the other trials and did not receive MTX before starting TNFα blocking therapy; patients enrolled in the adalimumab study had lower HAQ scores and therefore possibly a higher functional level than patients included in the etanercept and infliximab trials. CRP levels also seemed to be lower for the patients in the adalimumab study. Age, sex, rheumatoid factor status, and disease activity measured by tender and swollen joints were comparable across the three studies.

Adherence to therapy and the number of ACR50 responders with or without adjustment for non-responders are shown in Table 2. As shown in Table 2, there was a considerable risk difference (between intervention and control) for adherence to therapy, between infliximab standard dosage, infliximab double dosage, etanercept and adalimumab: 23.3%, 26.7%, 13.8% and 6.8%, respectively.

When using the absolute risk reduction for each of the three preparations to calculate the NNT, without further adjustment, the efficacy following each of the drugs with concomitant MTX was (presented in descending order): adalimumab, infliximab double dosage, etanercept, and infliximab standard dosage, respectively, with only minor differences when applying the ITT population instead of the PP population. Likewise, NNT values only changed slightly when the ITT estimated number of ACR50 responders was adjusted for the individual study control group event rate. These fully adjusted NNT values indicated an equal efficacy corresponding to

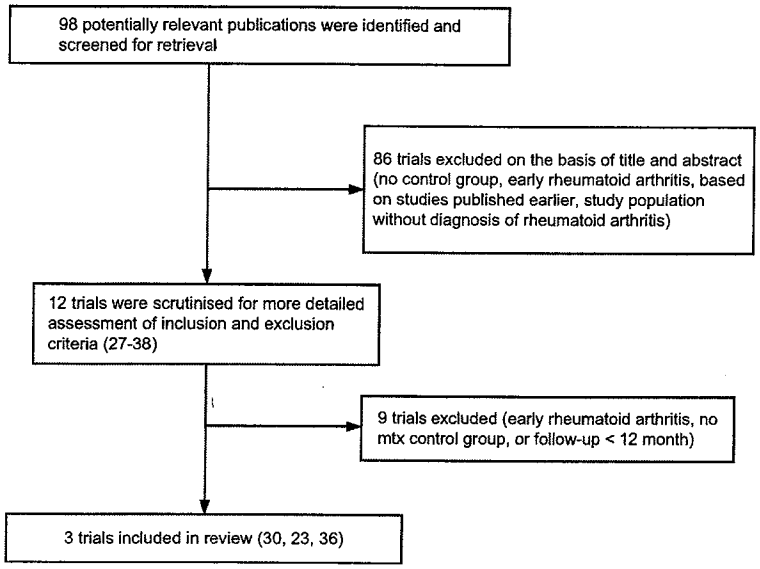

Figure 1. Flow chart of study selection performed by the two independent review groups.

Table 1. Demographic and clinical characteristics at baseline.

	Infliximab (36)			Etanercept (30)		Adalimumab (32)	
	3 mg/8 weeks	3 mg/4 weeks	Placebo	2×25 mg/week	Placebo	40 mg/2 weeks	Placebo
	Intervention N_{I1}=86	Intervention N_{I2}=86	Control N_C=88	Intervention N_I=231	Control N_C=228	Intervention N_I=207	Control N_C=200
Age, years	54±11	52±13	51±12	53±12	53±13	56±14	56±12
Female, %	81	77	80	74	79	76	73
Disease duration, years	10±8	9±8	11±8	6.8±5.4	6.8±5.5	11.0±9.2	10.9±8.8
Rheumatoid factor, % positive	84	80	77	76	71	82	90
Weekly MTX, mg	16±4	16±4	16±4	10*	10*	16.7±4.5	16.7±4.1
C-reactive protein, mg/dL	3.9±3.4	3.5±4.2	4.0±4.2	3.0±3.3†	2.6±2.8†	1.8±2.3	1.8±2.1
HAQ score	1.8±0.6	1.7±0.6	1.7±0.6	1.8‡	1.7‡	1.5±0.6	1.5±0.6
Number of tender joints	32±18	31±15	31±18	34.2±14.8	33.1±13.4	27.3±12.7	28.1±13.8
Number of swollen joints	22±12	21±11	21±12	22.1±11.3	22.6±10.7	19.3±9.8	19.0±9.5

HAQ, Health Assessment Questionnaire. Values are mean±SD, unless stated otherwise. *This was the median dosage for previous MTX use, patients included in this study did not have MTX from start, no SD was given for this value. †Values corrected by a factor of 10 compared to the original publication (30) after communication with the author. ‡No SD was given for these values.

four patients for adalimumab [95% confidence interval (CI) 3–6], etanercept (95% CI 3–6) and infliximab double dosage (95% CI 3–11), whereas standard dosage infliximab seemed only half as efficient with an NNT of 8 (95% CI 4–66) patients.

Discussion

This report, in agreement with other studies (18–20, 39, 40), supports equal efficacy of the three anti-TNF therapies; adalimumab, etanercept and infliximab. However, according to this study, infliximab might require an increased dosage level to reach comparable clinical impact. The two review groups independently selected the same three articles, and subsequently calculated NNTs among completers and randomized (14) patients together with control event-adjusted NNT values (2, 13, 17) by an indirect comparison strategy (15) using peer-reviewed data for adalimumab, etanercept, and infliximab at 12 months of follow-up. When comparing NNTs calculated for completers or randomized patients, the absolute NNT changed by up to 25% (see Table 2). This is noteworthy particularly because the NNT can be either increased or decreased depending on the level of adherence in either the control or the intervention group. This observation emphasizes the need for ITT adjustment and 95% CIs when calculating NNTs (14). Further adjustment for event rates in the control group only changed the

Table 2. Clinical efficacy (ACR50) and adherence to therapy data in rheumatoid arthritis patients treated with a TNFα blocking agent added to methotrexate (Intervention) compared to methotrexate alone (Placebo) at 1 year of follow-up.

	Infliximab (36)			Etanercept (30)		Adalimumab (32)	
	3 mg/8 weeks	3 mg/4 weeks	Placebo	2×25 mg/week	Placebo	40 mg/2 weeks	Placebo
Adherence to therapy, n (%)	63 (73.3)	66 (76.7)	44 (50.0)	193 (83.5)	159 (69.7)	159 (76.8)	140 (70.0)
ACR50 responders, n	18	29	7	159	98	86	19
% ACR50 responders (1-year completers)	28.6	43.9	15.9	82.4	61.6	54.1	13.6
% ACR50 responders (among randomized)	20.9	33.7	8.0	68.8	43.0	41.5	9.5
NNT (unadjusted; 1-year completers)	8 (4–35)	4 (3–9)	–	5 (4–9)		3 (2–4)	
NNT (unadjusted; among randomized)	8 (5–38)	4 (3–7)	–	4 (3–6)		4 (3–5)	
Control group event-rate (% among randomized)			8.0		43.0		9.5
NNT (control event-adjusted; among randomized)	8 (4–66) patients	4 (3–11) patients		4 (3–6) patients		4 (3–6) patients	

ACR, American College of Rheumatology (response criteria); TNFα, tumour necrosis factor alpha; NNT, number needed to treat; ITT, intention-to-treat.

NNT values slightly, with primary changes in CIs. This illustrates that the adjustment method used can only to some extent compensate for baseline differences between studies (16, 17). Therefore, this strategy cannot be applied uncritically for indirect treatment comparison in heterogeneous study designs. It should also be noted that the control event rate-adjusted NNT was originally developed for pooled studies (16, 17), giving more precision to the NNTs found based on a formal meta-analysis. Consequently, the results found in this review should be interpreted with caution. First, the comparison is based on only one study for each preparation. Second, it is difficult to eliminate confounders when comparing results from trials performed in different locations and in patients with possible differences in disease status. Moreover, clinically relevant differences at baseline were seen between the three studies (as presented in Table 1). Patients eligible for the etanercept study did not receive MTX before inclusion and their disease duration was shorter than patients enrolled in the infliximab and adalimumab trials. Etanercept patients therefore had a pronounced potential for responding to MTX and perhaps also to the TNFα blocking drug when entering the study. This difference should partly be balanced by using a control group receiving MTX. Furthermore, time- and baseline-adjusted NNT will compensate, to some extent, for the differences in baseline characteristics (15–17), which might complicate the interpretation of this indirect comparison of etanercept with infliximab and adalimumab. Thus, our method for comparison between these drugs is likely to be the only feasible way of obtaining such information, as direct comparisons of biologicals are too costly to perform in an RCT setting. Finally, subjects enrolled in the adalimumab trial had a trend towards lower HAQ values and CRP levels than subjects in the etanercept and infliximab trials. It is unclear how these differences in average baseline functional level (HAQ) might influence the outcome. Having a lower HAQ score has been shown to be advantageous by increasing adherence to therapy (41). However, it might also have been a disadvantage by giving less potential for change in the ACR50 values, which partially depend on the subjective score of functional status. Moreover, lower CRP levels have been shown to be a disadvantage by decreasing adherence to therapy (41), possibly because of less systemic inflammation and thus less potential for responding to anti-inflammatory drugs. Thus the NNT for adalimumab might have been even lower if the included patients had higher CRP levels at inclusion.

Given the unavoidable limitations of indirect comparisons, clinicians tend to put greater weight on factors other than apparent effectiveness when choosing between treatments (3). There are obvious differences between the three compounds concerning route of administration, dosage intervals, individual patient compliance, apparent prices, and expense for the outpatient clinic administering the drugs. Local guidelines take all these points into account when providing information regarding TNFα blocking treatment recommendations. However, the present NNT values might imply that infliximab requires more than standard dosages to achieve comparable effect levels, thus increasing the apparent cost of this preparation.

Additional evidence may arise from observational studies, and to facilitate interpretation of data from such trials we recently introduced a new approach to efficacy per se, entitled the LUNDEX (22). The LUNDEX is an index of the proportion (at a given time, T) of patients included (N_0) and remaining (N_T) on a particular therapeutic regimen with a corresponding adherence ratio (N_T/N_0), but also fulfilling certain response criteria (N_R) with a corresponding success ratio (N_R/N_{TR}): $\text{LUNDEX}_T=(N_T/N_0)\times(N_R/N_{TR})$. It should be noted that when clinical efficacy data are exhaustively recorded for all subjects at a given time, non-responder analysis and LUNDEX-adjusted efficacy responses are equal, suggesting that the LUNDEX is an unbiased estimator in observational studies. We therefore advocate using the LUNDEX adjustment when calculating the NNT from observational studies. Accordingly, this LUNDEX-adjusted NNT can be calculated as the inverse of the difference between the LUNDEX indices on trial. The use of the LUNDEX adjustment in observational trials or non-responder analyses in RCTs not only provides statistical properties but also increases the clinical transparency, with the presentation of the comparable true number of patients who benefit from the intervention.

In conclusion, our indirect comparison of available 12-month trials using TNFα blocking agents in combination with MTX indicates equal efficacy of adalimumab, etanercept, and infliximab, as one in four patients with established RA experience an ACR50 response compared to MTX as monotherapy. Infliximab might require an increased dosage to reach an efficacy comparable to adalimumab and etanercept. In this study, adjusting the NNT for non-responders and for the control group event rate only changed the values slightly. Finally, we hope that these detailed efficacy data, from three mutually independent high-quality trials, can and will be translated into clinically useful evidence-based rheumatology.

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Expert Opinion

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Abatacept: the first T lymphocyte co-stimulation modulator, for the treatment of rheumatoid arthritis

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Rheumatoid arthritis (RA) is a multisystem autoimmune disease, of unknown aetiology with high morbidity and significantly increased mortality. Over recent years, the introduction of targeted therapies with biologic agents have made major inroads to the outcomes in RA.The first such agents developed were TNF-α inhibitors. However, despite their high efficacy, up to 30% patients fail to respond adequately, or develop adverse reaction to TNF-α inhibitors. This suggests that other pathological mechanisms are involved, in addition to those mediated by TNF-α. Abnormal T-cell function has long been thought to play a key role in the pathogenesis of RA, stimulating both the production of pro-inflammatory cytokines and recruitment of other inflammatory cells, resulting in joint destruction and systemic disease. Abatacept, the first of a group of T-cell co-stimulation modulators, targeting T-cell activation, has recently been licensed for use in RA and shows promise as a useful drug to treat this major disabling disease.

Keywords: abatacept, biologics, CTLA-4, CTLA4-Ig, rheumatoid arthritis, TNF inhibitors

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1. Introduction

Rheumatoid arthritis (RA) is a multisystem, autoimmune, inflammatory disorder of unkown aetiology, characterised by systemic inflammation and a destructive synovial proliferation in affected joints. It affects ~ 1% of the population and ~ 40% of affected individuals develop severe joint damage and experience disability at 20 years from disease onset. In addition, RA is associated with increased cardiovascular mortality [1], partly linked to RA disease activity [2]. Seropositive RA patients have an increased cardiovascular mortality, even in the early stages of the disease [3]. The aim of treating RA is to induce and then maintain remission. This requires early and dynamic therapy with agents that are able to suppress the underlying response, hence reducing damage and improving outcome [4].

Though small-molecule drugs or disease-modifying antirheumatic drugs (DMARDs), typified by methotrexate have provided effective disease activity suppression at low cost, in most RA patients, they have failed to halt radiographic progression of disease. Also, a small but significant proportion of patients continue to have a high disease activity. Recent advances in understanding the pathological processes that underlie RA, have led to major improvements with the development of biologic targeted therapies. The TNF-α inhibitors were the first such agents and, especially when used in combination with methotrexate have radically improved the treatment outcomes for RA – not only by diminishing the destructive nature of RA but also improving survival [5]. However, ~ 30% of patients with RA fail TNF-inhibitor therapy because of inefficacy or adverse reactions [6]. There remains, therefore, an unmet therapeutic need in RA and a requirement to discover new theraputic approaches and treatment options.

The pathological importance of cytokines in RA is underlined by the clinical effectiveness of the TNF inhibitors. However, there is much evidence to suggest that the cellular specific immune system, in the form of T cells also plays a prime role in the disease [7]. The largest genetic component for susceptibility to RA lies in the class II MHC, responsible for presenting antigens to T cells; there is a massive infiltration of $CD4^+$ T cells in inflamed joints [8-10] and many animal models of inflammatory arthritis are mediated by T cells. Activation of T cells may potentially drive the inflammatory process in RA, leading to recruitment and activation of other immune system cells and the secretion of pro-inflammatory cytokines and release of metalloproteinases and other inflammatory mediators that ultimately lead to bone and cartilage damage and destruction [11]. Effective T-cell activation requires interaction with antigen-presenting cells (APC) that deliver a combination of signals; signal 1, which is mediated via the T-cell receptor and the second co-stimulatory signal, so-called as it stimulates the T-cell in conjunction with the antigen. [12,13]. The appropriate delivery of both signals will activate T cells, however signalling through the T-cell receptor alone, in the absence of signal 2, may lead to T-cell anergy. The best-characterised T-cell co-stimulation pathway involves the CD28 receptor, which binds the two co-stimulatory molecules CD80 (B7-1) and CD 86 (B7-2) [14,15].

Abatacept (Orencia®; Bristol-Myers Squibb) is the first biological, selective co-stimulation modulator, modulating the T-lymphocyte stimulation by blocking the interaction of CD-28 with its ligand on APC. Abatacept binds with higher affinity to the CD80/86 on the APC, so the second signal required for T-cell activation does not occur. Its primary effect is on naive T cells as memory T cells are less dependent on the second signal. Abatacept does not deplete T cells.

It was approved by the FDA for use in RA in 2005 and has just received a license in Europe. Abatacept inhibits the inflammatory response by blocking the activation of helper T cells and in Europe is licensed for the treatment of moderate-to-severe active RA in adults who have had an insufficient response or intolerance to other DMARDs including at least one TNF inhibitor.

2. Overview of market

Abatacept will compete with other biological DMARDs. Initially, this will be in situations of TNF inhibitor failure, although eventually it may compete earlier on in the theraputic timeline. Three TNF inhibitors are currently licensed: infliximab (Remicade; Schering-Plough), etanercept (Enbrel; Wyeth) and adalimumab (Humira; Abbott). Rituximab (Mabthera; Roche) a chimeric depleting anti-CD20 (B cell) monoclonal antibody, originally developed to treat B-cell lymphomas, obtained a supplemental biological license in October 2006 for use in RA patients inadequately responding to anti-TNF agents.

It is likely that abatacept will primarily compete with rituximab in patients that respond inadequately to (or are intolerant to) TNF inhibitors. Abatacept can be used as a monotherapy or in combination with other non-biological DMARDs and is administered by monthly intravenous infusion. The projected sales for abatacept could reach US $1 billion by year 2009/10 [6].

3. Biological therapies

3.1 TNF inhibitors

These agents neutralise the action of TNF-α and hence reduce inflammation. Etanercept is a human soluble TNF-α p75 receptor construct administered subcutaneously at weekly intervals. Infliximab is a chimeric monoclonal anti-TNF-α antibody administered by intravenous infusion at 8-week intervals, whereas adalimumab is a humanised monoclonal antibody given subcutaneously, 14 days (or in resistant cases, weekly) apart. Infliximab is licensed for coadministration with methotrexate. The others can be used as monotherapy but all of the TNF-α inhibitors are most effective when used alongside methotrexate. This form of treatment is most effective in up to 70% patients with RA [6].

3.2 Anti-CD20 antibody

Rituximab is a depleting monoclonal anti-CD20 antibody that targets peripheral B cells. It has been licensed in the US and Europe since 2006 for use in RA in patients, who fail or inadequately respond to anti-TNF agents. Rituximab is administered in a cycle of two intravenous infusions, 14 days apart. Each cycle may result in a disease-suppressing effect that lasts on an average 9 months. Coadministration of methotrexate is associated with a more prolonged response. Recent studies have shown that repeated B-cell depletion is effective and relatively well tolerated [16].

4. Traditional disease-modifying antirheumatic drugs

Methotrexate is the most commonly used generic, non-biological DMARD.

Leflunomide (Arava, sanofi-aventis) has comparable efficacy and side effect profile to methotrexate in treating RA. However, clinically, there is a higher frequency of drop out due to mild yet troublesome side effects. Methotrexate, used in combination with biologic antirheumatic agents is known to increase their efficacy without significant additional side effects [17,18].

5. Introduction to abatacept

Abatacept is the first of the group of T lymphocyte co-stimulation modulators. It was approved for use in RA by the FDA in December 2005 for patients with moderate-to-severely active RA who have had an inadequate

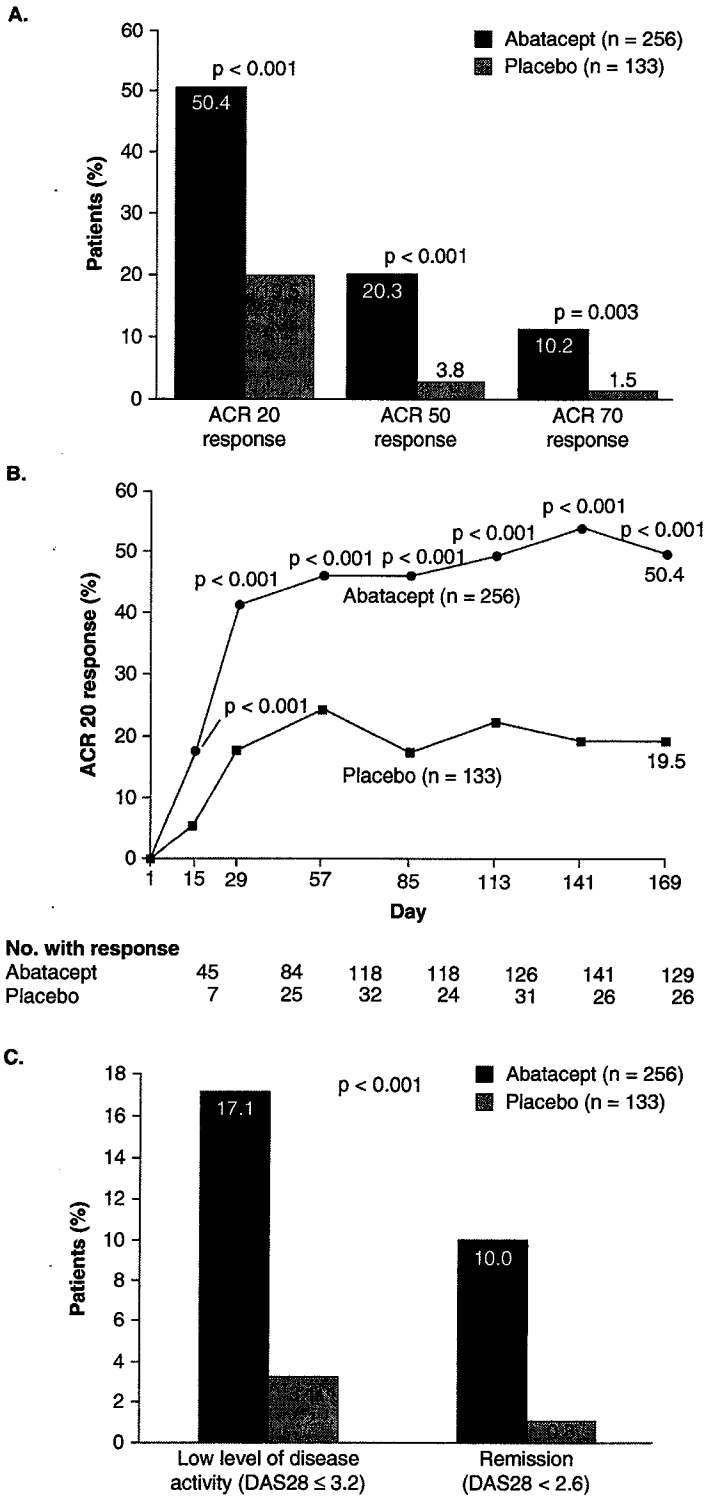

Figure 1. Results from the abatacept trial in the treatment of anti-TNF inadequate responders trial. A. ACR 20, 50 and 70 response in patients on abatacept and placebo. **B.** ACR 20 response over 6-month period in patients with abatacept and placebo. **C.** Comparison of percentage of patients achieving low disease activity (as measured by DAS-28) or remission between patients on abatacept and placebo.

Table 1. Mean radiographic changes over 12 months.

Parameter	Abatacept/Mtx n = 391	Placebo/Mtx n = 195	p value*
Total sharp score	1.21	2.32	0.012
Erosion score	0.63	1.14	0.029
JSN score	0.58	1.18	0.009

*Based on non-parametric analysis.
JSN: Joint space narrowing; Mtx: Methotrexate.

response to or failed one or more DMARDs, such as methotrexate or TNF-α inhibitors. In this respect, the US license permits the usage of abatacept before TNF inhibitors, in contrast to the license awarded in Europe, where TNF inhibitor(s) should be used first.

5.1 Chemistry

Abatacept is a soluble protein consisting the extracellular domain of human cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), fused with the modified Fc portion of a human IgG1 antibody (**Figure 1**). It has an apparent molecular weight of 92 kDa and is manufactured using recombinant DNA technology on a human cell line. Commercially it is available as a lyophilised powder, which needs to be reconstituted with 10 ml sterile water prior to intravenous injection.

5.2 Pharmacodynamics

Abatacept blocks the interaction between APC and T cells leading to the inhibition of T-cell activation (**Figure 2**). Abatacept (CTLA4-Ig) binds to CD80 and CD86, thus blocking co-stimulation through CD28 [19-22]. It thus modulates T-cell proliferation and the many downstream consequences of this including secretion of TNF-α, γ-IFN, IL-1 and IL-6.

5.3 Pharmacokinetics and metabolism

Following a 10 mg/kg dose of abatacept intravenously, in normal subjects, a peak concentration of 292 mg/l (range 175 – 427 mg/l) was achieved. The elimination half-life was 16.7 days (range 12 – 23 days), total clearance 0.23 ml/h/kg (range 0.16 – 0.30 ml/h/kg) and the volume of distribution at steady-state was 0.09 l/kg (range 0.06 – 0.13 l/kg). Monthly 10 mg/kg intravenous doses of abatacept in RA patients revealed similar pharmacokinetics [20]. The clearance increased with increase in weight but was not altered by age, gender, NSAIDs, DMARDs or anti-TNF agents [23].

5.4 Clinical efficacy

5.4.1 Phase II-A

A multi-centre, multinational study was performed to determine the safety and preliminary efficacy of co-stimulatory blockade using abatacept and LEA29Y (Belatacept) (both in doses of 0.5.2 or 10 mg/kg) in RA patients who had been treated unsuccessfully with at least one DMARD. Abatacept, LEA29Y or placebo was administered intravenously to 214 RA patients. They received 4 infusions of the study medication or placebo on days 1, 15, 29 and 57 and response was assessed at day 85. The primary end point of the study, ACR 20 increased in a dose-dependent manner with abatacept (23, 44 and 53%) as compared with placebo (34%). Abatacept was found to be safe and was well tolerated [24]. Patients were then randomly assigned to 2 mg/kg or 10 mg/kg of abatacept or placebo for a further 6 months. Clinical improvement at 6 months was evaluated using ACR20, 50 and 70 responses [25]. Belatacept has not been further pursued for use in RA.

5.4.2 Phase II-B

This trial investigated the efficacy and safety of abatacept 2 mg/kg in combination with etanercept 25 mg biweekly in patients with active RA during a 1-year, randomised, placebo-controlled, double-blind phase, followed by an open-label, long-term extension. At 1 year, the combination of abatacept and etanercept was associated with serious adverse events including infections, with limited additional clinical benefit [26,27].

5.4.3 Phase III

The Phase III trial programme included three double-blind randomised placebo-controlled studies.

The abatacept in inadequate responders to methotrexate trial compared abatacept in combination with methotrexate or methotrexate alone. This was carried out over a 2-year period, of which the first year included a double-blind phase with subsequent open-label extension phase. Patients on abatacept with methotrexate, in comparison with patients on methotrexate alone showed a better ACR response and reduction in radiographic progression (**Table 1**) [28].

In the abatacept trial in the treatment of anti-TNF inadequate responders trial, patients with active RA who responded inadequately to TNF-α inhibitor therapy, after treatment of at least 3 months, were randomly assigned in a 2:1 ratio to receive abatacept or placebo on days 1, 15, 29, and every 28 days thereafter for 6 months, in addition to at least one non-biologic DMARD. Patients discontinued anti-TNF therapy prior to randomisation. The rates of ACR 20 responses and improvement in functional disability, as reflected by scores for the health assessment questionnaire (HAQ) disability index were assessed [29]. Results are shown in **Figure 1**.

The abatacept study of safety in use with other RA therapies studied the safety of abatacept as compared with placebo when used in conjunction with non-biologic and biologic DMARDs. In this trial, patients were randomised 2:1 to receive abatacept (at a fixed dose approximating 10 mg/kg by weight) or placebo for a period of 1 year. Abatacept and placebo groups exhibited similar frequencies

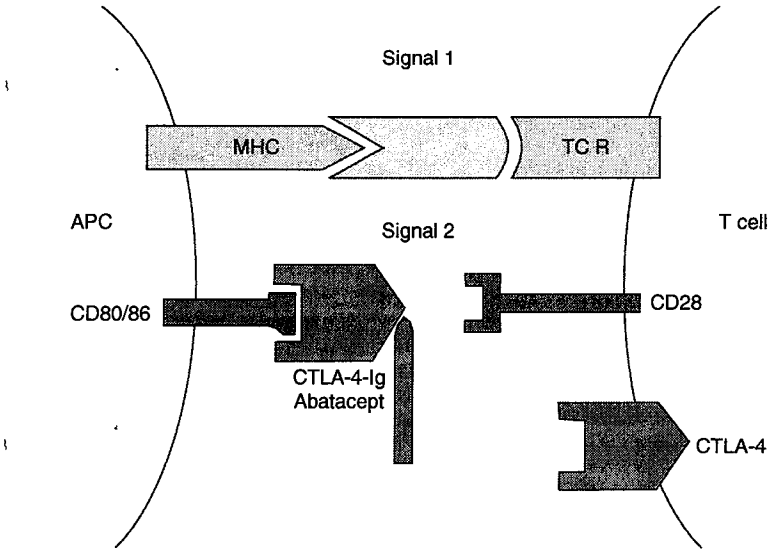

Figure 2. Mechanism of action of abatacept.
APC: Antigen-presenting cell; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; TCR: T-cell receptor.

of adverse events (90 and 87%, respectively), serious adverse events (13 and 12%, respectively) and discontinuation due to adverse events (5 and 4%, respectively). Abatacept in combination with non-biologic DMARDs was well tolerated with no increase in the infection risk, and improved physical function and physician and patient reported outcomes. However, abatacept in combination with biologic therapies was associated with an increase in the rate of serious adverse events including infections, with little additional clinical benefits [30]. Hence, combination of abatacept with other biological DMARDs is not recommended.

5.4.4 Indications

Abatacept has been approved by the FDA to reduce signs and symptoms in RA, induce a major clinical response, inhibit progression of structural damage and improve physical function in adult patients, with moderate-to-severely active RA, who have failed to or have had inadequate response to one or more DMARDs or TNF antagonists [19].

6. Other therapies on horizon

There are many different drugs targeting a variety of immunological pathways under development for treatment of RA. Most agents in later phase clinical trials are biologics; however, small-molecule drugs, for example p38 mitogen-activated protein kinase inhibitors, are also being developed. These will also compete with abatacept. One example of an addition to an existing class is the PEGylated anti-TNF blocker, cetrolizumab Pegol (Cimzia, UCB). This TNF inhibitor has demonstrated efficacy in Phase III clinical trials in RA and offers potential advantages over present TNF inhibitors in dosing regimen and manufacturing cost. Golimumab, another fully humanised anti-TNF-α IgG1 monoclonal antibody that targets and neutralises membrane-bound and soluble forms of TNF-α has completed its Phase II trial, showing that combination of golimumab and methotrexate is significantly superior to methotrexate alone in improving disease activity in RA [31]. Humanised IL-6 antibody (Tocilizumab; Roche) has completed the first Phase III clinical trial and shows promise as an inhibitor of a different yet potentially equally important cytokine. Data from that study showed that patients on tocilizumab showed a significantly reduced radiographic progression preventing joint erosion and joint space narrowing as compared with non-biologic DMARD therapy [32].

7. Safety and tolerability

7.1 Adverse reactions

Abatacept is generally well tolerated. The commonest adverse events recorded in clinical studies were naso-pharyngitis, headache, nausea, cough and upper respiratory tract infection. Sero-conversion to anti-CTLA-4 Ig antibodies has occurred in 0 – 1.3% patients, with no clinical significance [23].

7.2 Drug interactions

The clearance of abatacept was not altered with concomitant use of DMARDs, NSAIDs or anti-TNF agents [20]; however, the concomitant use of biologic agents with abatacept increased the risk of infections without increasing efficacy and hence is not recommended [23,30].

7.3 Regulatory affairs

Abatacept obtained the FDA approval in 2005, for use in moderate-to-severe RA, failed on or inadequately treated with methotrexate or anti-TNF therapy [19].

The European Medicines Agency has recently approved use of abatacept in Europe, after a favourable report from the Committee for Medicinal Products for Human Use [33,34]. Unlike the US it is licensed for use in moderate-to-severe RA patients who have failed on at least one biological agent. Abatacept is currently being evaluated by the UK national institute of clinical excellence and a decision expected by early 2008.

8. Conclusion

Despite many recent advances in the therapy of RA, typified by the development of TNF inhibitors, there remain unmet theraputic needs. Abatacept is a prototype T-lymphocyte co-stimulation modulator, modulating the activation of T lymphocytes, thus reducing cytokine production (such as TNF-α, IL-1 and -6), inflammation and damage. Abatacept is effective when used alone or in conjunction with non-biological DMARDs (such as methotrexate), in patients who have failed on methotrexate monotherapy. Its efficacy and side effect profile is comparable to that of the TNF inhibitors. In addition, abatacept can be used in patients who have failed on anti-TNF therapy. Use in conjunction with other biological therapies, however, is not advocated. Early clinical trials have shown benefits in health-related quality of life compared with placebo [35,36]. As a new drug, the long-term efficacy and safety profile are not available. Further clinical trials and postmarketing follow-up on patients treated with abatacept is necessary to gain a more complete understanding of its true efficacy and risks.

9. Expert opinion

The pathogenesis of RA is complex, involving dysfunction in many levels of the immune system: specific, innate, cellular and humoral. Understanding the importance of pro-inflammatory cytokines and, hence, the development of TNF inhibitor therapy led to major improvements in the outcome in RA. Yet these drugs are ineffective in 30% of patients and some data suggests that up to 50% of RA patients have to stop TNF inhibitor therapy within 5 years. Whether this reflects the development of neutralising antibodies (as is likely for the monoclonal antibody drugs) or the development of alternative pathophysiological mechanisms independent of TNF blockade remains uncertain [6]. The complex immunological nature of RA and the chronicity of the disease suggest that the ability to selectively target different pathways involved in the pathogenesis is important for the long-term suppression of the pathological inflammatory response.

Abatacept is the prototype T-cell co-stimulatory modulator with proven efficacy in RA before or after exposure to TNF inhibitors in clinical trials. The mechanism of action of this drug, the modulation of T-cell activation, differs significantly from all licensed agents. By modulating T-cell activation, abatacept has the potential to affect multiple downstream pathways to reduce inflammation [37]. In addition to playing a key role in the activation of naive T cells in early disease [38], co-stimulation is likely to play a role in the pathogenesis of established long-standing disease.

In the short-term abatacept is likely to be used in TNF failures as these drugs are effective and now have 10 years of safety data. However, as experience with abatacept in the clinic increases, it is possible that it might be used earlier on in the therapy of RA with the fact that no single drug is effective for all patients and that efficacy for any drug may wane with time, indicates an important role for abatacept and for other future agents that inhibit T-cell activity in the treatment of RA.

Declaration of interest

The authors have no conflict of interest to declare and no fee has been received for preparation of the manuscript.

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